Structural and functional analyses of an ABC protein in isoquinoline alkloid-producing plant cells(Dissertation_全文)

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Contents

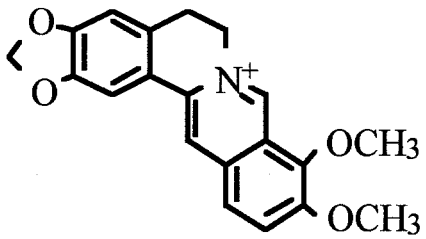
Abbreviations

ABC	ATP-binding cassette
cDNA	complementary deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycoltetraacetic acid
HPLC	high performance liquid chromatography
kDa	kilo Dalton
MDR	multidrug-resistance protein
MRP	multidrug-resistance-related protein
NBF	nucleotide binding fold
4-NQO	4-nitroquinoline *N*-oxide
PCR	polymerase chain reaction
PDR	pleiotropic drug resistance
PVDF	polyvinylidene difluoride
5'RACE	rapid amplification of the 5' end of cDNA
SDS	sodium dodecylsulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TMD	transmembrane domains
Tris	tris (hydroxymethyl) aminomethane
UTR	untranslated region

Introduction

Plant cells produce a large variety of alkaloids which have diverse chemical structures and biological activities. Some of them are used as medicines, such as anticancer drugs, analgesics, relaxant, and antimalarial drugs. They also play important roles in plants as endogenous biological barriers to protect them from pathogens or herbivores due to their strong antimicrobial, antifungal activities and other cytotoxicities as well. For instance, berberine, a yellow benzylisoquinoline alkaloid, which is used as a bitter stomachic and an anti-diarrhetic, shows strong antimicrobial activity toward both Gram-positive and Gram-negative bacteria as well as other microorganisms (Fig. 0-1) (Iwasa *et al.*, 1998).

In many cases, the chemical structures of the alkaloids are too complex to be chemically synthesized on a commercial base. Thus, plants are still main source materials for the supply of alkaloids in the market. Although plants are renewable resources, some species are on the brink of extinction, and therefore the supply of these valuable alkaloids is getting more difficult to meet the increasing demands. With the development of enzymology and molecular biology on the plant alkaloid biosynthesis, we are now capable of altering the alkaloid productivity and the composition of alkaloids in plants by genetic engineering of the alkaloid biosynthesis to breed new



Berberine

Biological activities:

- Antibacterial activity
- Anti-inflammatory activity
- Monoamine oxidase inhibition
- Dopamine receptor inhibition
- DNA intercalation

Pharmaceutical use:

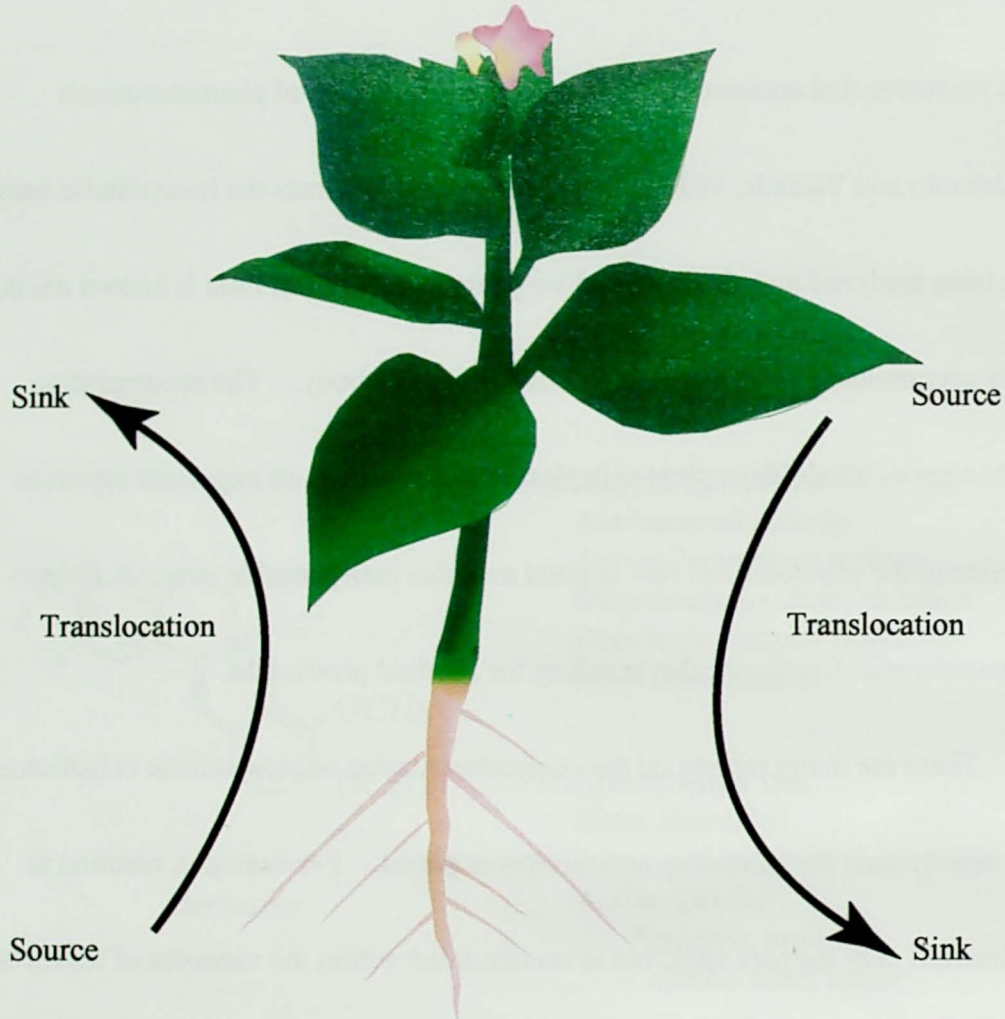
- Bitter stomachic
- Intestinal antiseptic
- Antineoplastic*

*putative prodrug against brain tumor

Fig. 0-1 Structure, biological activities, and pharmaceutical use of berberine.

plant resources that accumulate increased amounts of desired pharmaceuticals (Hashimoto and Yamada, 1994, Sato *et al.*, 2001). Whereas the biosynthetic enzymes have been analyzed and characterized very intensively so far, little is known about how plants accumulate and transport alkaloids in the plant body. The accumulation mechanism of alkaloids in plant cells tissues and organs is an important aspect to understand the physiological role in plant and also indispensable research field to achieve the effective molecular breeding for alkaloid production.

There are many reports on the compartmentation of biosynthetic cells/tissues and sink cells/tissues for secondary metabolites in plants. For example, nicotine is synthesized near the root apex, but is accumulated within the vacuoles of leaves in *Nicotiana* species (Hashimoto and Yamada, 1994), and senecionine *N*-oxide, a pyrrolizidine alkaloid, is synthesized in the root, and translocated to be accumulated in the vacuoles of inflorescences in *Senecio* species, such as *S. vulgaris* or *S. vernalis* (Hartmann, 1999) (Fig. 0-2). Several reports describe the subcellular transport mechanism of secondary metabolism into the vacuoles (Deus-Neumann and Zenk, 1986, Mnede and Wink, 1987), however, little is known how alkaloids are translocated from the source tissue to the sink tissue, and how alkaloids are loaded in and unloaded from the vascular tissue in the long distance transport.



Plant	Alkaloid	Source organ	Sink organ
Nicotiana species	Nicotine	Root	Leaf
Senecio. vulgaris or S. vernalis	Senecionine *N*-oxide	Root	Inflorescence
Atropa belladonna *Hyoscyamus niger*	Hyoscyamine Scopolamine	Root	Leaf
Coptis japonica	Berberine	Root	Rhizome

Fig. 0-2 Translocation of alkaloids.
Alkaloids are synthesized in the source organ, and translocated to the sink organ to be accumulated.

In *Coptis japonica* (Ranunculaceae), a perennial medicinal plant grown in Asian countries, berberine is highly accumulated in the rhizome as the main alkaloid. However, the genes for the biosynthesis of berberine are specifically expressed in the root tissue (Fujiwara *et al.*, 1993), where only a low level of berberine is detected (Fig. 0-3). This suggests that berberine is transported from the root tissue after the biosynthesis to the rhizome, then unloaded there to be highly accumulated. Cultured *C. japonica* cells produce berberine and accumulate it exclusively in the vacuoles (Sato *et al.*, 1990, 1994). Furthermore, exogenous berberine that is added to the culture medium is actively taken up by *C. japonica* cells (Sato *et al.*, 1990, 1994), and is also accumulated in the cell vacuoles (Sato H. *et al.*, 1993). The preliminary studies to identify the transporter of berberine in *C. japonica* cells suggested that an ABC (ATP-binding cassette) protein was involved in berberine transport in this plant cells (Terai and Yazaki unpublished data).

The ABC protein superfamily is one of the largest protein families known in prokaryotes and eukaryotes, i.e. from bacteria to human (Higgins, 1992; Henikoff *et al.*, 1997). Most, but not all, are membrane proteins, which are often called "ABC transporters" and they are active in the transport of a broad range of substances across membranes. ABC proteins possess one or two ATP-binding cassettes, which are also

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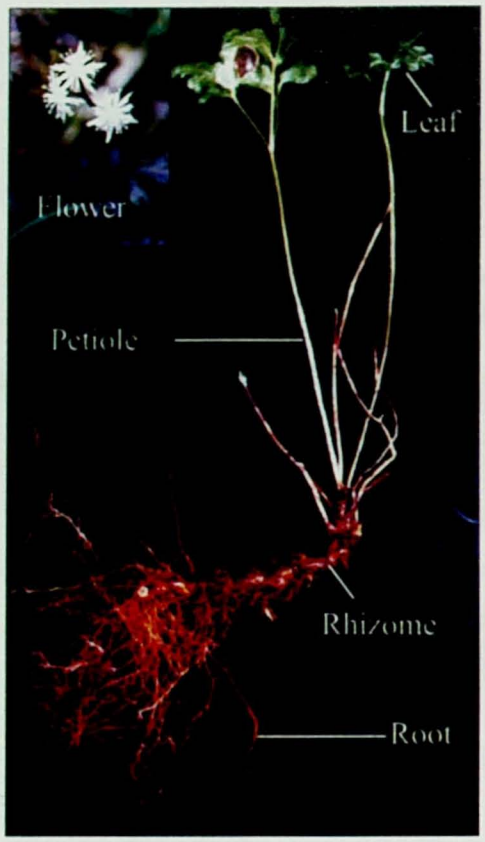
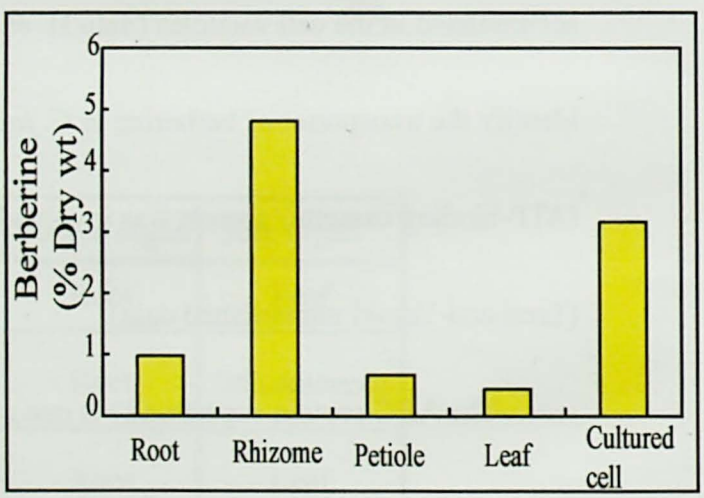


(B)

(C)



(Ref.; Fujiwara *et al.*, 1993)

Fig. 0-3 *Coptis japonica* and berberine accumulation
(A) *Coptis japonica* MAKINO var. dissecta NAKAI
(B) Cultured cells of *C. japonica*
(C) Berberine amounts in *Coptis* tissues and cultured cells

designated nucleotide binding folds (NBF), where an identity of 30-40% over a region of about 200-400 amino acid residues can be found. This domain contains three highly conserved sequence motifs. These are called Walker A box and Walker B box that are separated by approximately 120 amino acids (Walker *et al.*, 1982), and an ABC signature motif situated between the two Walker boxes (Fig. 0-4).

ABC transporters consist of two pairs of basic structural elements; hydrophobic integral transmembrane domains (TMDs) and NBFs oriented towards the cytoplasm. The TMDs are considered to form the pathway for solute movement across the phospholipid bilayer, and appear to determine or at least contribute to the substrate selectivity of the transporter. In bacteria, the four domains can reside on different independent polypeptides, whereas bacterial genes encoding fusions of all four domains have, to date, rarely been found (Linton and Higgins., 1998; Braibant *et al.*, 2000). In contrast, in plants – as in animals and yeast – such proteins are encoded by conserved gene families. The so-called "full-size ABC transporters" can be subdivided into two groups according to their topology: multidrug-resistance proteins (MDRs, also known as PGPs standing for P-glycoproteins) and multidrug-resistance-related proteins (MRPs), which show a structure of TMD1-NBF1-TMD2-NBF2. The mirrored topology (NBF1-TMD1-NBF2-TMD2) can be found for PDR (pleiotropic drug resistance) and

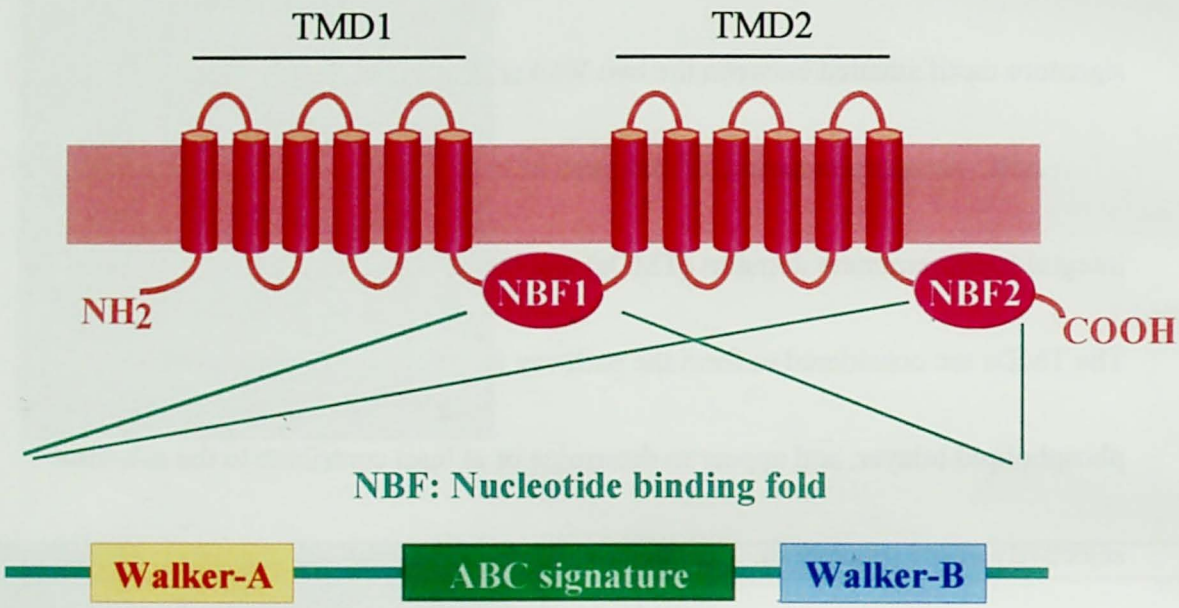


Fig. 0-4 Schematic drawing of a typical ABC transporter
ABC transporter consists of transmembrane domains (TMD) and NBF. NBF contains three conserved sequence motifs: an ATP-binding site consisting of a Walker A box and a Walker B box separeted by approximately 120 amino acids and, between the two boxes, an ABC signature.

ABC1-like genes (Higgins, 1992; Bungert *et al.*, 2001).

Plant ABC transporters comprise one of the most active research fields in recent years. Based on the results of the *Arabidopsis* genome project, this plant contains a large number of this family members, e. g. 129 open reading frames in *A. thaliana* genome (Sánchez-Fernández *et al.*, 2001; Martinoia *et al.*, 2002). This gene count far outstrips those for the human genome and for any other organisms' genome sequenced to date. The human genome is estimated to encode a mere 48 ABC proteins (http://www.humanabc.org); those of *Caenorhabditis elegans* (19,000 ORFs; http://www.proteome.com/databases/WormPD/WormPDsearch-long.html), *Drosophila melanogaster* (13,600 ORFs; http://flybase.bio.indiana.edu/), and *Saccharomyces cerevisiae* (6,000 ORFs; http://mips.gsf.de/proj/yeast/CYGD/db/index.html) only 58, 55, and 31, respectively.

The first ABC transporter isolated from plants was an *mdr*-like gene from *A. thaliana* (AtPGP1; Dudler and Herting, 1992). Further experiments showed that AtPGP1 was localized in the plasma membrane and involved in hypocotyl elongation in light-grown seedlings (Sidler *et al.*, 1998). Observation of glutathione conjugate uptake into plant vacuoles (Martinoia *et al.*, 1993) triggered the identification of vacuolar ABC transporters in plants. They belong to MRPs or GS-X pumps and are

considered to be involved in the intracellular sequestration of toxins or xenobiotics (Rea *et al.*, 1998; Rea, 1999; Theodoulou, 2000). Among several *mrp*-like genes identified in *Arabidopsis*, five have been characterized in more detail. AtMRP1, AtMRP2, and AtMRP3 encode functional GS-X pumps and transport xenobiotics and endogenous substances, such as chlorophyll catabolites, into the vacuole (Lu *et al.*, 1997, 1998; Tommasini *et al.*, 1998). Expression of AtMRP4 was found to be induced by herbicide safeners (Sánchez-Fernández *et al.*, 1998). AtMRP5 transport glutathione conjugates and glucuronide conjugate. In barley, the vacuolar uptake of glucoside derivatives has been characterized and suggested to be catalyzed by an ABC transporter (Klein *et al.*, 1996).

Recent results indicate that the function of this protein family is not restricted to detoxification processes. *Nicotiana plumbaginifolia* ABC1 (NpABC1) (Jasinski *et al.*, 2001) and *Spirodela polyrrhiza* TUR2 (SpTUR2) (van den Brule *et al.*, 2002), which are PDR-like transporter, are reportedly localized in the plasma membrane. Several experimental data suggest that these proteins are involved in the excretion of secondary metabolite, sclareol, which is antifungal diterpene playing a role in plant defense. Plant ABC proteins have been demonstrated to participate in chlorophyll biosynthesis (Møller *et al.*, 2001), formation of Fe/S clusters (Kushnir *et al.*, 2001), stomatal

movement via probably ion fluxes (Gaedeke *et al.*, 2001); hence they may play a central role in plant growth and developmental processes.

As shown above, there were only few studies on the ABC proteins involved in the transport of endogenous metabolite, such as secondary metabolites. In order to characterize such transport mechanisms, I used *C. japonic*a as a model plant, with which the transport mechanism for the endogenous alkaloid, berberine, was studied. The advantage of this plant is that a cell culture system was established, which have high ability to produce berberine and grows much rapidly than the intact plant (Sato and Yamada, 1984). In chapter I, I examined the involvement of ABC protein in the berberine transport and the existence of three ABC proteins in the cells. In Chapter II, I isolated *mdr*-like genes (*Cjmdr1* and *Cjmdr2*) from *C. japonica* cells and examined their tissue specific expression. In Chapter III, I analyzed the berberine transport activity of CjMDR1 using heterologous expression systems. In Chapter IV, I showed that CjMDR1 was localized in the plasma membrane of *C. japonica* cells and specifically expressed in the xylem tissue in *C. japonica* rhizome.

Chapter I

Biochemical characterization of ABC protein in berberine transport in cultured *Coptis japonica* cells

Berberine, a benzylisoquinoline alkaloid occurring *Berberis*, *Phellodendron*, *Mahonia*, *Nandina*, *Thalictrum*, and *Coptis* species, is an important pharmaceutical with antibacterial, stomachic and anti-inflammatory activity. The biosynthetic pathway to berberine has been clarified, and 13 enzymes that are involved in stepwise conversion of two molecules of L-tyrosine to one molecule of berberine have been identified. Cultured *C. japonica* cells produce berberine and accumulate it exclusively in the vacuoles (Sato *et al.*, 1990, 1994). Furthermore, exogenously added berberine is actively taken up by *C. japonica* cells (Sato *et al.*, 1990, 1994), and is also accumulated in the cell vacuoles (Sato H. *et al.*, 1993). In this chapter, I report that the uptake of berberine by *C. japonica* cells required intracellular ATP. Further analyses using specific inhibitors of ABC proteins have suggested that a member of ABC transporters was involved in the transport of berberine into *Coptis* cells.

Results

Characterization of berberine uptake by C. japonica *cells*

As shown in Fig. 1-1, *C. japonica* cells constantly absorbed berberine added to the medium, and berberine had almost completely disappeared from the medium 12 hours after the addition. The berberine content in the cells treated with berberine was 4.82 μmol/0.25 g fresh weight, whereas that in the control cells was 4.10 μmol/0.25 g fresh weight. The increase of berberine coincided with the amount of berberine added to the medium, 0.75 μmol, which indicated that the berberine was taken up by the cells from the medium and stably accumulated, as reported previously (Sato *et al.*, 1990, 1994).

Inhibition of berberine uptake

Several compounds which inhibit the activity of various transporters were examined by addition to the medium. Fig. 1-2 shows a summary of the effect of the inhibitors tested. Vanadate, azide, cyclosporine A, and nifedipine strongly inhibited the berberine uptake. Vanadate is a membrane ATPase inhibitor (Ambudkar *et al.*, 1992). Azide is known to reduce the internal ATP when cells are treated (Wigler and Patterson 1994). On the other hand, both cyclosporine A and nifedipine are inhibitors

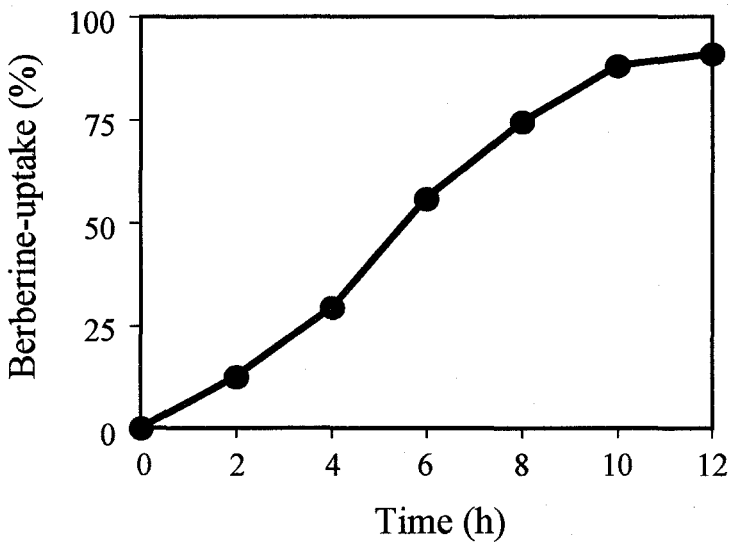


Fig. 1-1 Berberine uptake by cultured *C. japonica* cells.
Berberine was added 14 days after inoculation at an initial concentration of 250 μM.
The value 100% means the complete uptake of the berberine added to the medium.

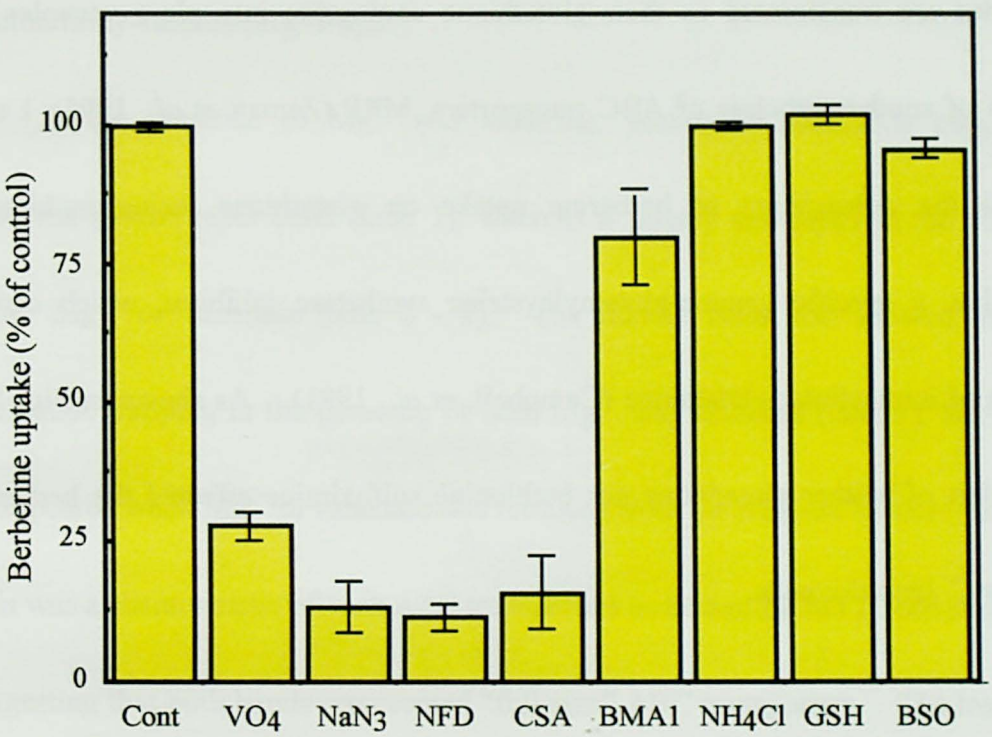


Fig. 1-2 Effects of various inhibitors on the berberine uptake.
Cont, control; VO_4, vanadate (1mM); sodium azide (100 μM); NFD, nifedipine (50 μM); CSA, cyclosporin A (100 μM); BMA1, bafilomycin A1 (1 μM); NH_4Cl, ammonium chloride (10 mM); GSH, glutathione (100 μM); BSO, buthionine sulfoximine (1 mM).

of an ABC transporter, human MDR1, which functions as a drug efflux pump in human cancer cells (Ueda at al. 1987). These data suggested that an ABC transporter might be involved in the uptake of berberine from the medium. Because some secondary metabolites are translocated as their glutathione conjugates into plant vacuoles by members of another subclass of ABC transporters, MRP (Zaman *et al.*, 1995), I also examined the dependency of berberine uptake on glutathione, using buthionine sulfoximine, a specific gamma-glutamylcysteine synthetase inhibitor, which causes depletion of intracellular glutathione (Campbell, *et al.*, 1991). As shown in Fig. 1-2, the addition of neither glutathione nor buthionine sulfoximine affected the berberine uptake by *C. japonica* cells.

Detection of ABC proteins in C. japonica *cells*

A photoaffinity labeling experiment was carried out to detect ABC proteins in *C. japonica* cells, and its interaction with berberine was investigated. ABC proteins show ATPase activity, and the catalytic sites are known to be of low affinity and specificity for nucleotides. The binding affinity for nucleotides of the ABC protein greatly increased in the presence of vanadate (Urbatsch *et al.*, 1995). A catalytic site produces a stable form of the inhibited ABC protein by trapping of nucleotide with vanadate

which is labeled specifically with 8-azido-[α-^{32}P] ATP by UV irradiation (Taguchi *et al.*, 1997). When this vanadate-induced nucleotide trapping method was applied to the crude cell membranes of *C. japonica*, three bands of 120 to 150 kDa were photoaffinity-labeled (Fig. 1-3).

In the presence of Mg^{2+} and vanadate, prominent nucleotide trapping was observed in the upper band (lane 1), whereas it largely decreased in the absence of either Mg^{2+} or vanadate (lane 2 - 4). The middle band also showed the strong nucleotide trapping in the presence of both Mg^{2+} and vanadate (lane 1), but it almost disappeared when Mg^{2+} or vanadate was removed from the reaction mixture (lane 2 - 4). This was a characteristic of such ABC transporters as human MDR1 (Ueda *et al.*, 1997), suggesting that both bands represented "full-size" ABC transporters. The lower band, however, required Mg^{2+} ion for ATP-binding, but still strongly bound ATP even in the absence of vanadate (lane 1, 2). This suggested that this protein did not hydrolyze ATP to ADP, in a similar way as a member of ABC proteins, sulfonylurea receptor 1 (Ueda *et al.*, 1997).

The addition of berberine to the reaction mixture apparently reduced the nucleotide trapping of the upper and middle bands in a dose-dependent manner (lane 5 – 8), whereas the lower band was unaffected. These data suggested that both the upper

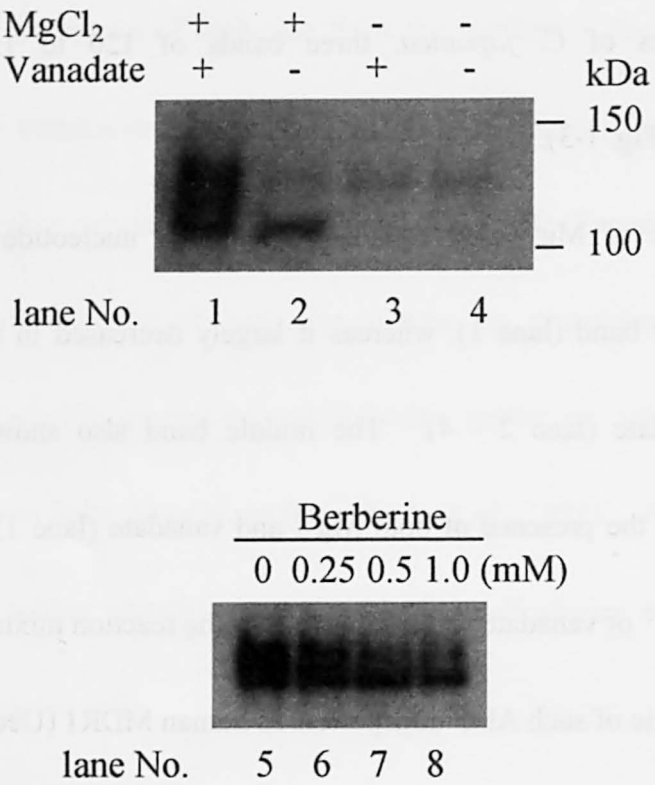


Fig. 1-3 Photoaffinity labeling of ABC proteins in membranes of *C. japonica* cells with 8-azido-[α-^{32}P] ATP. Membrane proteins from cultured cells of *C. japonica* were incubated with 10 μM 8-azido-[α-^{32}P] ATP, in the presence or in the absence of 3 mM $MgCl_2$ and 200 μM orthovanadate for 10 min at 37°C (lane 1 - 4). In lane 5 - 8, berberine was added to the incubation mixture at concentration of 0 to 1.0 mM. Proteins were photoaffinity-labeled with UV irradiation after removal of unbound ligands and analyzed by SDS-PAGE.

and middle bands interacted with berberine.

Discussion

When berberine was exogenously added to *C. japonica* cultures, it was actively absorbed by the cells and disappeared from the medium (Fig. 1-1). The added berberine was transported into the vacuoles and stably accumulated (Sato *et al.*, 1990, 1994; Sato H. *et al.*, 1993).

The transport systems for secondary metabolites in plant cells are often reported to be energy-dependent (Yamamoto *et al.*, 1987; Deus-Neumann and Zenk 1986; Mende and Wink 1987). Indeed, the uptake activity of berberine by *C. japonica* cells was very sensitive to the membrane ATPase inhibitor, vanadate. Furthermore, various inhibitors of human MDR1 decreased the berberine transport into the cells (Fig. 1-2). Human MDR1, a representative of ABC transporter, was discovered in human cancer cells as a large membrane protein responsible for multiple drug resistance against anti-cancer drugs. Its expression confers on the cancer cells multidrug resistance by effluxing the chemicals of divergent structures, which results in the decrease of their intracellular concentration (Ueda *et al.*, 1987).

The results of vanadate-induced nucleotide trapping experiments suggested the

existence of ABC transporters in *C. japonica* cells, and two of the three bands (the upper and the middle) showed the characteristic properties of the drug efflux pump, human MDR1. The interaction of these two putative ABC transporters with berberine suggested that they were involved in the transport of berberine in this plant cell (Fig. 1-3).

The involvement of ABC transporters in the uptake of berberine from the medium was speculative, but endogenously biosynthesized berberine might be transported in a different way. Zenk *et al.* (1985) reported that the biosynthetic berberine precursors in *Fumaria capreolata* cells were localized in membrane vesicles and then the final product berberine was transported to the vacuolar matrix. The uptake of various alkaloids by isolated plant vacuoles has been demonstrated to be highly specific for plant species and is energy-dependent (Deus-Neumann and Zenk, 1984, 1986; Mende and Wink, 1987; Wink and Mende, 1987). The transport processes of solutes across the vacuolar membrane of higher plants are complicated, and many membrane proteins of divergent classes are involved (Martinoia, *et al.*, 2000). ABC proteins could be responsible for alkaloid accumulation in plant vacuoles, as is the case for other metabolites (Lu *et al.*, 1998; Marrs *et al.*, 1995; Klein *et al.*, 1996) (Fig. 1-4).

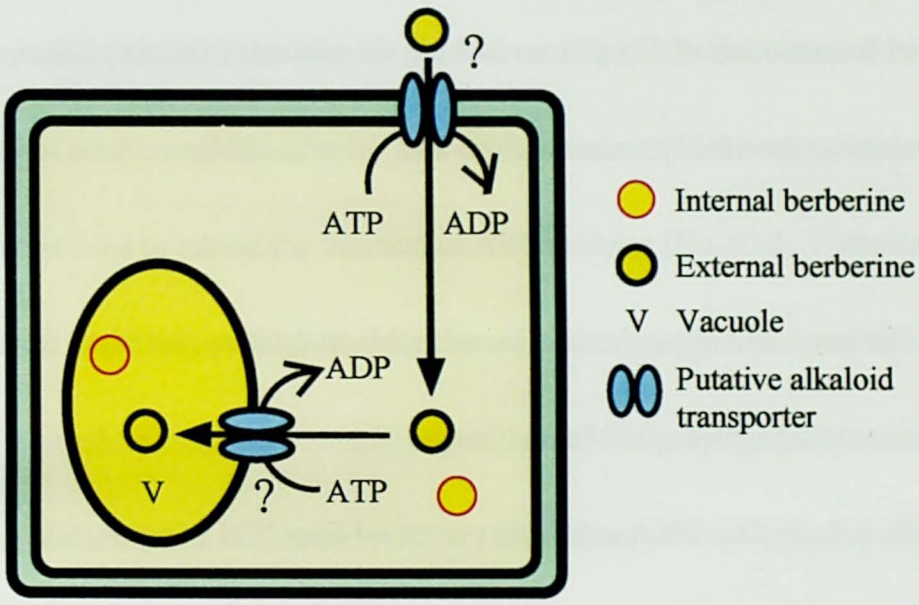


Fig. 1-4 A model for active transport of berberine in *Coptis japonica* cell
Berberine is actively taken up by *C. japonica* cell, and accumulated in the cell vacuole. ABC transporter might be involved in the transport of berberine at the plasma membrane, tonoplast membrane, or both.

Materials and Methods

Cultured Cells.

High berberine-producing cultured *C. japonica* cells, which were originally induced from rootlets of *C. japonica* Makino var. *dissecta* (Yamabe) Nakai, were maintained as described previously (Sato and Yamada, 1984).

Chemicals.

Berberine and other chemicals used in this study were purchased from Wako Pure Chemicals (Osaka, Japan) or Nakalai Tesque (Kyoto, Japan). The 8-azido-[α-^{32}P] ATP (specific activity 740 GBq/mmol) was purchased from ICN Biochemicals.

Berberine uptake by cultured cells.

Cultured *C. japonica* cell were harvested by filtration and a 0.25 g fresh mass was inoculated into 3 ml of the culture medium in which the cells had grown (conditioned medium) in a test tube. Berberine chloride was added to the medium at 250 μM, and the cells were incubated on a rotary shaker at 25°C. Every 2 h, a 150 μl aliquot of the medium was mixed with 350 μl water, and centrifuged at 15,000 g for 5 min. The concentration of berberine was determined by measurement of the absorbance of the supernatant at 420 nm (ε =3,330). Experiments were done in triplicate.

Inhibitor experiments.

Prior to the addition of berberine, *C. japonica* cells (0.25 g) were treated with inhibitors (vanadate, 1 mM; azide, 100 μM; nifedipine, 50 μM; cyclosporine A, 100 μM; bafilomycinA1, 1 μM; NH_4^+ 10 mM; glutathione 100 μM; buthionine sulfoximine, 1mM) in 3 ml of conditioned medium for 1 h. These concentrations were chosen from the normal range used to inhibit the function of ABC proteins (Hu *et al.*, 1996; Klein *et al.*, 1996; Lu *et al.*, 1998). After the addition of berberine (final concentration 250 μM) the uptake was measured at 25°C. Sodium vanadate was depolymerized before use according to the method of Goodno (1979). Quinidine, nifedipine, and cyclosporin A were dissolved in DMSO solution, and 10 μl each was added to the medium. In the control, 10 μl of DMSO or H_2O was added. DMSO did not affect the berberine uptake or cell viability at this concentration. The viability of the cells was confirmed by the staining with neutral red in the conventional assay. Alternatively, cell growth was measured after the treated cells were washed and recultivated.

Photoaffinity labeling of ABC proteins with 8-azido ATP.

Fresh cells (1.0 g) were homogenized in 3 ml of 0.1 M potassium phosphate buffer (pH 6.5) containing 10 mM dithiothreitol (DTT) and 0.1 g polyvinylpolypyrrolidone using a mortar and pestle. After removal of cell debris by

centrifugation at 3,000 g, the supernatant was gel-filtered with PD-10 (Amersham-Pharmacia) to change the buffer to 0.1 M Tris-HCl (pH 7.4) containing 10 mM DTT, and then the membrane fraction was recovered by centrifugation at 20,000 g for 30 min at 4°C. Membrane proteins (20 μg) were incubated in a buffer mixture composed of 10 μM 8-azido-[α -^{32}P] ATP, 2 mM ouabain, 0.1 mM EGTA, 3mM $MgSO_4$, 40 mM Tris-HCl (pH 7.5), and 0.2M *ortho*-vanadate in a total volume of 8 μl for 10 min at 37°C (Senior *et al.*, 1995). The reactions were stopped by the addition of 400 μl of ice-cold TGM buffer (40 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, and 1mM $MgSO_4$), and the membrane proteins were sedimented by centrifugation (15,000 g, 10 min, 4°C) to separate unbound ATP. Pellets were washed with fresh ice-cold Tris-EGTA buffer, then irradiated with UV light at 254 nm (5.5 mW/cm^2) in 8 μl of TGM buffer for 5 min on ice. Samples were electrophoresed on a 7% SDS-polyacrylamide gel, and autoradiographed. The radioactivity trapped by ABC protein was analyzed with a radioimaging analyzer BAS 2000 (Fuji Film Co.). Experiments were repeated three times.

Chapter II

Molecular cloning of *mdr*-like genes from cultured *Coptis japonica* cells

In Chapter I, several experiments to identify the transporter of berberine in *C. japonica* cells showed that the uptake of berberine by *C. japonica* cells depended on the ATP level, and several inhibitors of human MDR1, a representative ABC protein responsible for multiple drug resistance of cancer cells (Ueda *et al.*, 1987), significantly suppressed berberine uptake from the medium. These results suggested that an ABC protein was involved in berberine transport in this plant cells.

The ABC protein superfamily is one of the largest protein families known in prokaryotes and eukaryotes (Higgins, 1992; Henikoff *et al.*, 1997). Most, but not all, are membrane proteins, which are often called "ABC transporters" and they are active in the transport of a broad range of substances across membranes. ABC proteins possess one or two ATP-binding cassettes, which are also designated nucleotide binding folds (NBF). This domain contains three highly conserved sequence motifs, Walker A box, Walker B box, and an ABC signature motif.

In this Chapter, I report the molecular cloning of *mdr*-like genes, *Cjmdr1* and *Cjmdr2*, by RT-PCR from *C. japonica* cells, as well as their primary structure and tissue

expression.

Results

Isolation of cDNAs of Cjmdr1 *and* Cjmdr2

ABC proteins possess one or two NBF, where an identity of 30-40% over a region of about 200-400 amino acid residues is found. This domain contains three conserved sequence motifs, i.e. a Walker A box, a Walker B box, and an ABC signature motif situated between the two Walker boxes. Four degenerate primers were designed to isolate a cDNA fragment corresponding to NBF domain of *mdr*-like protein, and RT-PCR was done as described in Materials and Methods. To obtain a full-length cDNA clone, 5' and 3' RACE using a cDNA amplification kit (Clontech) was used (Fig. 2-1). Two full-length cDNAs were isolated and designated *Cjmdr1* and *Cjmdr2* (*Coptis japonica* multidrug resistance). *Cjmdr1* and *Cjmdr2* were ca. 4.2 kb long and encoded a putative polypeptide composed of 1289 and 1292 amino acids, respectively. They shared 80% and 82% identities each other on the nucleotide level and amino acid level, respectively, whereas the DNA sequences of both 5'- and 3'-UTR (untranslated region) were only 10 to 21% identical. A protein database search revealed that CjMDR1 and 2 polypeptides belonged to the *mdr*-like gene family, but not to another

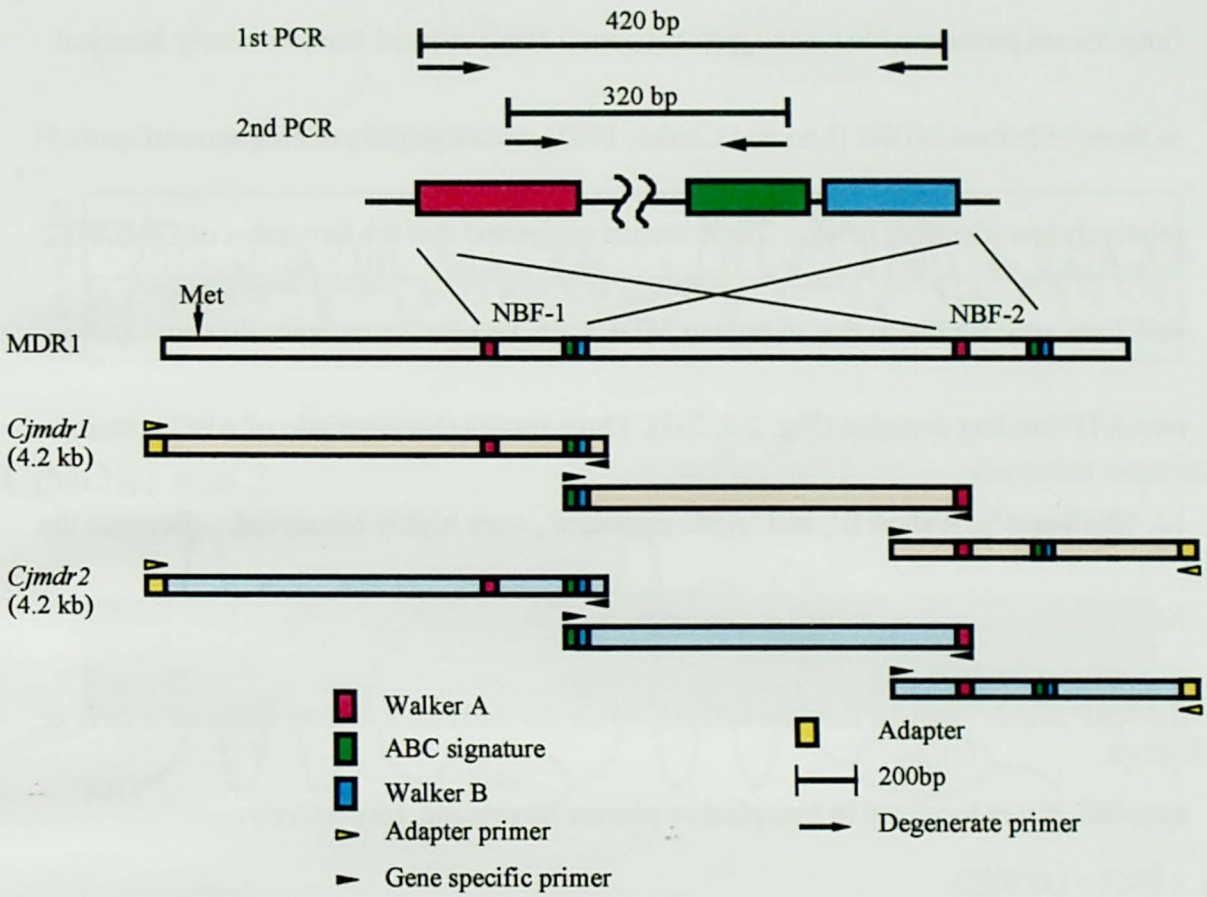


Fig. 2-1 Cloning strategy for *mdr*-like cDNA by PCR
NBF domain of *mdr*-like protein were isolated by nested PCR using degenerate primers designed from the highly conserved amino acid sequences. Using two specific primers corresponding to the internal sequences of these DNA fragments, RT-PCR was performed. To obtain a full-length cDNA clone, 5' and 3' RACE were done.

subfamily of ABC protein, MRP (Rea, 1999). Their hydrophobicity profiles calculated by a Kyte and Doolittle plot and the program on The SOSUI system (http://sosui.proteome.bio.tuat.ac.jp/sosuimenu0.html) showed features nearly identical to those of human MDR1 (Loo and Clarke, 1993), although the proteins showed relatively low identity (38%). These results suggested that the structures of CjMDR1 and 2 are very similar to that of human MDR1, i.e. two transmembrane domains and two ATP-binding domains (Fig. 2-2, 2-3). Three motifs characteristic of ABC protein, i.e. 'Walker A', 'Walker B', and 'ABC signature', were highly conserved. Because the NBF domains function as ATPase as demonstrated in MDR, and other ABC proteins, the NBF side is likely to face the cytosol, while the other side will be vacuolar matrix or apoplast, if it is localized in tonoplast or plasma membrane, respectively.

Cjmdr1 *and* 2 *expression in* C. japonica *plant and berberine accumulation*

The expression of *Cjmdr1* and *2* in the *C. japonica* cells and various organs of intact *C. japonica* plant was monitored by Northern blot hybridization with 3'-UTR as the probe for specific detection of these molecular species. In the cultured *C. japonica* cells, time-course experiment showed that the expression level of *Cjmdr1* and *2* remained almost constant which was similar pattern as that of the main alkaloid

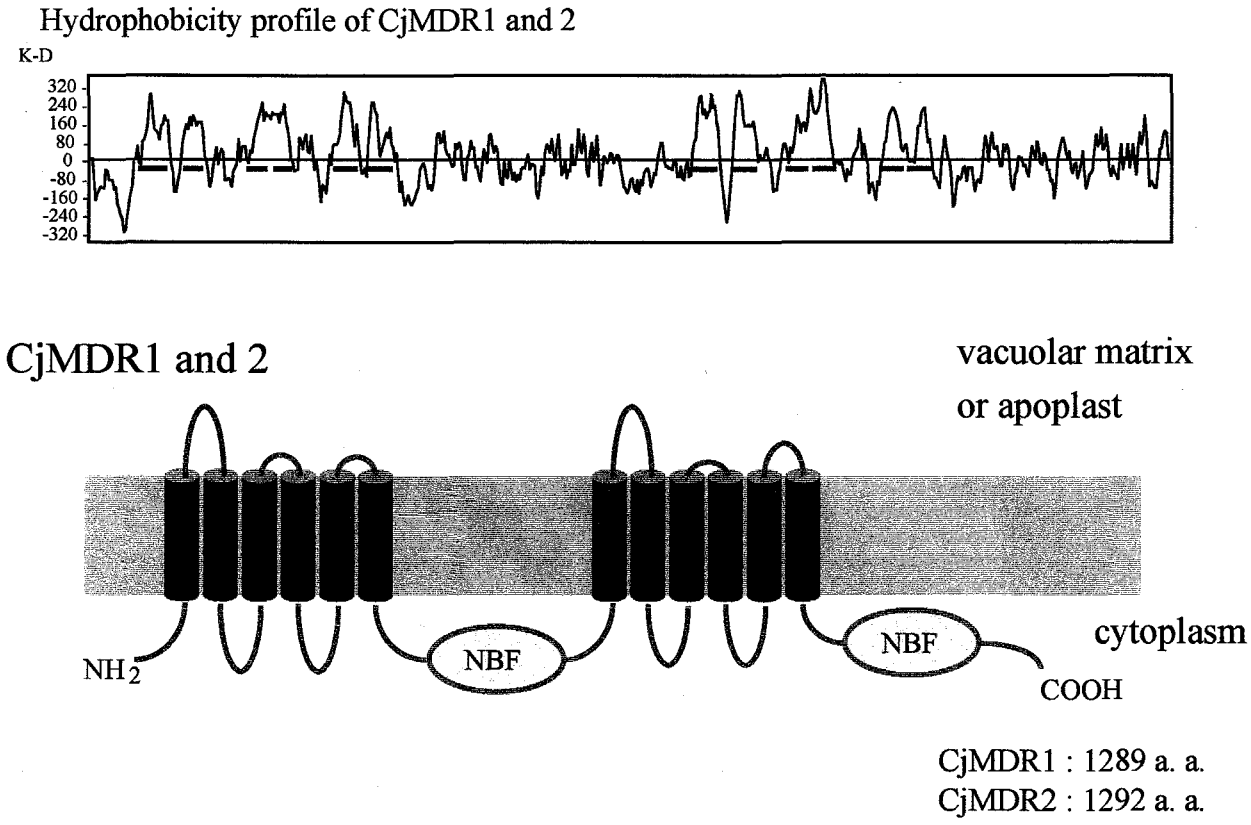


Fig. 2-2 Hydrophobicity profiles and putative topology of CjMDR1 and 2. Hydrophobicity profiles of CjMDR1 and CjMDR2 calculated by a Kyte and Doolittle plot suggested that the structures of CjMDR1 and 2 had twelve transmembrane α-helixs (underlined). CjMDR1 and 2 had two transmembrane domains and two ATP-binding domains (NBF). NBF is considerd to be located in the cytosol.

Fig. 2-3 Amino acid alignment of CjMDR1 and CjMDR2.
Enclosed boxes indicate identical amino acid residues. Putative transmenbrane domains containing the predicted transmembrane helices are underlined. Walker A, ABC signature, and Walker B are color underlined.

berberine content in the cells, although the mRNA level appeared to be higher in the growth phase than the stationary and lag phase (Fig. 2-4). However, a clear preference was observed in the organ-specific expression analysis as shown in Fig. 2-5. The highest accumulation of *Cjmdr1* and *2* mRNA was detected in rhizome of the intact plant, where the main accumulation site of berberine (Fujiwara *et al.*, 1993), whereas only a low level of transcripts was detected in other organs. Since other members of the plant *mdr*-like gene family have not shown such a clear organ specificity (Dudler and Herting, 1992; Wang *et al.*, 1996; Davies *et al.*, 1997; Sasaki *et al.*, 2002), these were the first plant *mdr*-like gene products that were expressed preferentially in rhizome, and demonstrated a correlation between gene expression and alkaloid accumulation in a plant organ.

In the phylogenetic relationship of plant *mdr*-like proteins, the sequences of CjMDR1 and 2 were very similar to those of AtPGP4 and AtPGP21 from *A. thaliana* (Fig. 2-6). However, functions of these *Arabidopsis* ABC proteins are yet unclear.

Discussion

Based on the conserved region of ABC protein, two full lengths of *mdr*-like genes, *Cjmdr1* and *Cjmdr2*, were isolated by nested RT-PCR from alkaloid-producing

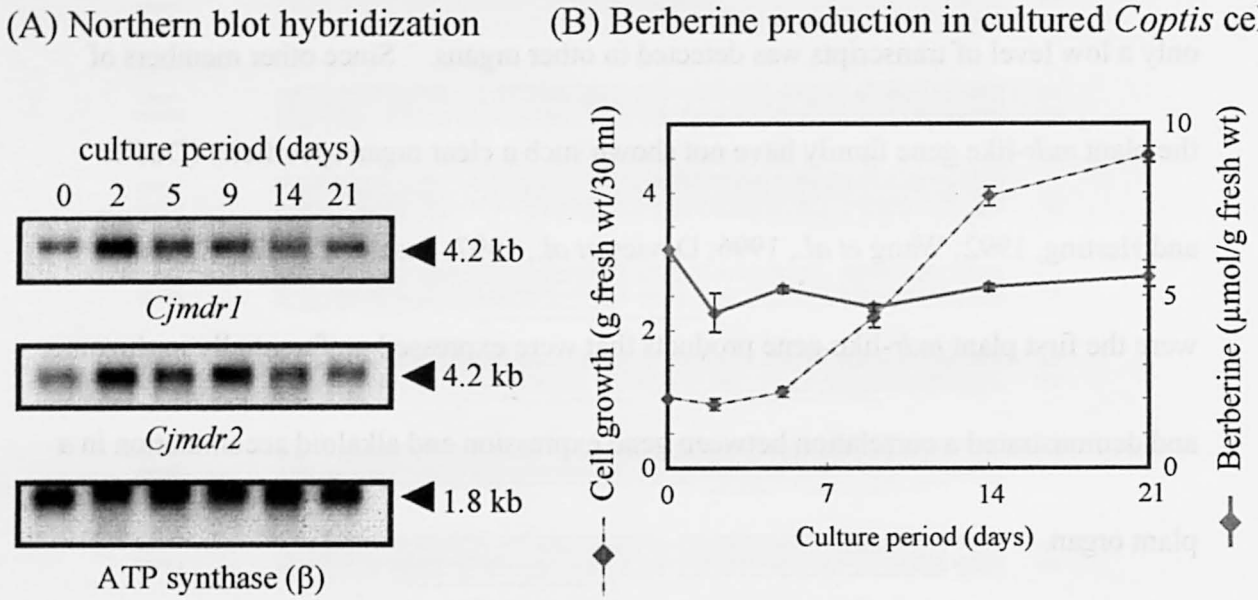


Fig. 2-4 Northern hybridization of *Cjmdr1*, *2* (A), and berberine accumulation in cultured *C. japonica* cells (B). The cultured cells were harvested 2, 5, 9, 14, 21 days after inoculation, 3 μg of poly $(A)^+$ RNA was isolated and loaded on formamide-containing agarose gel. As the hybridization probe, 3'-untranslated region of *Cjmdr1* (200 bp) and *Cjmdr2* (200 bp) were used. ATP synthase (β subunit) was used as the load control. Berberine content in the cells was monitored by HPLC analysis.

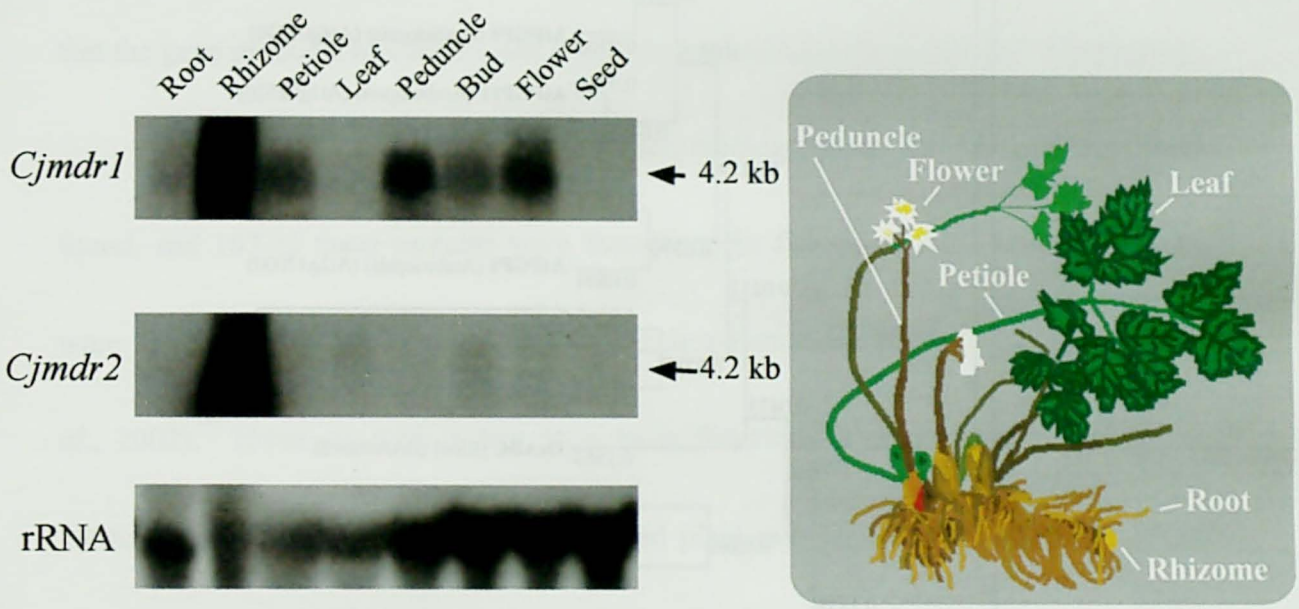


Fig. 2-5 Northern analysis of *Cjmdr1* and *2* in *C. japonica* plant.
Organ-specific expression of *Cjmdr1* and *Cjmdr2* in the intact plant of *C. japonica* detected by Northern hybridization (probe: 200 bp fragment of *Cjmdr1* or *Cjmdr2* 3'-untranslated region). Total RNA (10 μg) was isolated from each organ and separated on formamide-containing agarose gel. 18S rRNA was used as the load control.

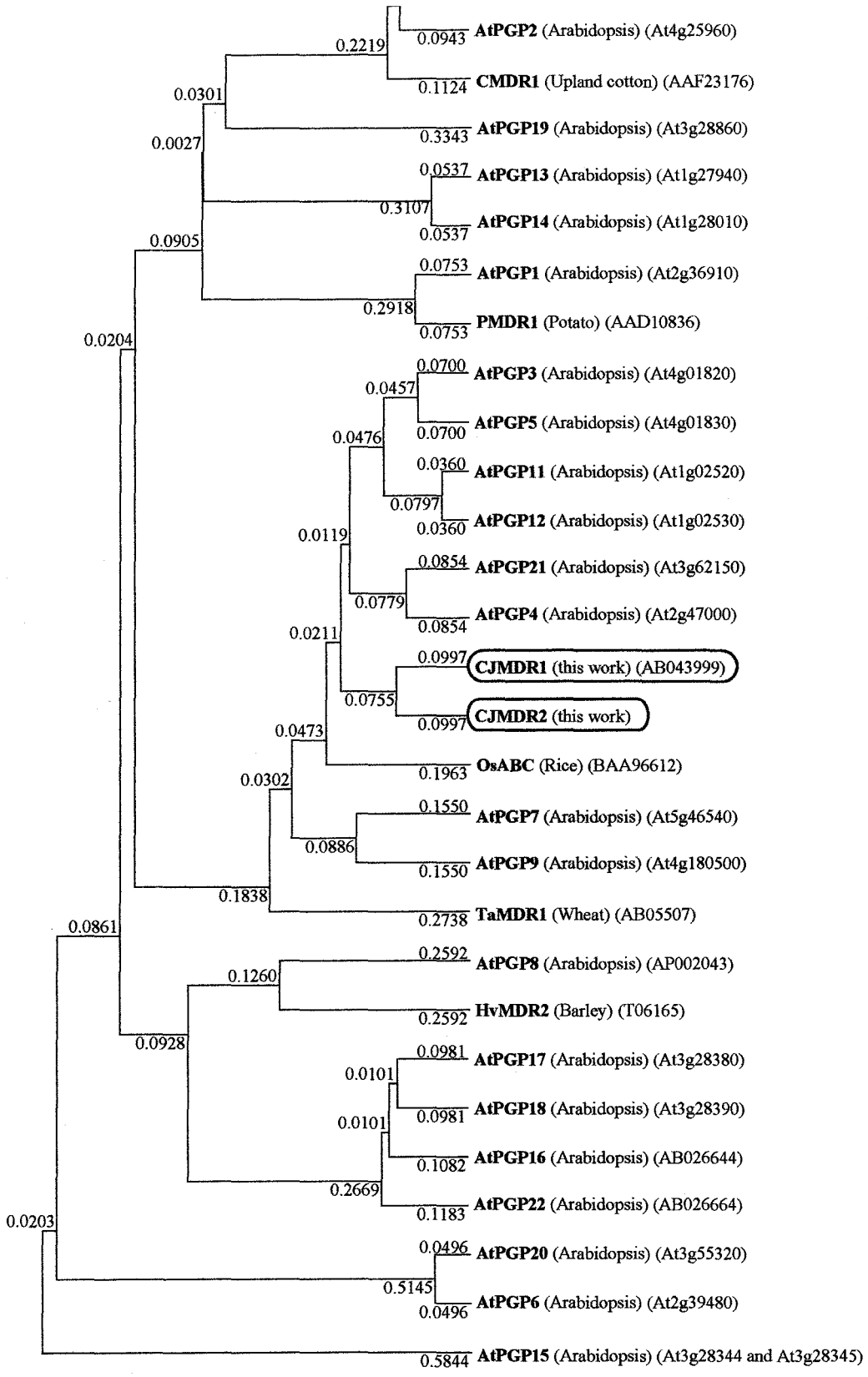


Fig. 2-6 Phylogenetic relationship of plant MDR-like proteins.
MDR-like protein sequences obtained from GenBank™ were used to build the tree. MIPS-Code or Accession numbers are indicated in parenthesis. Sequneces were aligned by the multisequence alignment program in GENETYX-MAC ver. 1.1 (Software Development, Inc) using the UPGMA (unweighted pair group maximum average) metho

C. japonica cells. They were ca. 4.2 kb nucleotides in length showing open reading frames composed of 1289 and 1292 amino acids, respectively. Northern analysis of the intact plant showed a clear preference in its expression in the rhizome, where alkaloids were highly accumulated in comparison with other organs, which suggested that the gene products of *Cjmdr1* and *2* were involved in the accumulation of berberine.

In the genome of *A. thaliana*, 129 open reading frames for ABC proteins were found, and 103 of these proteins were thought to be functional ABC transporters that were integrated in membrane via TMD (Sánchez-Fernández *et al.*, 2001; Martinoia *et al.*, 2002). However, only a few have been functionally characterized (Rea *et al.*, 1998; Theodoulou, 2000). The first isolated plant *mdr*-like gene was the AtPGP1 of *Arabidopsis* (Dudler and Hertig, 1992). This full-length ABC protein was localized in the plasma membrane, and was known to be involved in light-dependent hypocotyl cell elongation (Sidler *et al.*, 1998). Its heterologous expression in yeast has suggested that this ABC protein might transport ATP (Thomas *et al.*, 2000), and experiments using *Arabidopsis* mutants suggested that AtPGP1 was required for the normal auxin distribution and auxin-mediated plant development (Noh *et al.*, 2001). Other MDR-like ABC proteins have been isolated from potato (Wang *et al.*, 1996), barley (Davies *et al.*, 1997), and wheat (Sasaki *et al.*, 2002), but their substrates and

subcellular localizations are still unknown.

ABC transporter forms often a large gene family. In *A. thaliana* genome, the existence of 22 members of MDRs, which constitute the second largest subfamily of ABC protein superfamily and the largest full size ABC transporter subfamily. In the genomic Southern blot using 1.4 kb *Cjmdr1* fragment as a probe, in which two NBFs were eliminated, one strong and five to nine faint bands were detected in *C. japonica* (data not shown). This suggested that *mdr*-like genes also composed of a gene family in the genome of *C. japonica*.

Materials and Methods

Chemicals.

Unless otherwise stated, the purest chemicals were obtained and used as described in Chapter I.

Isolation of full-length *mdr*-like gene from cultured *C. japonica* cells.

Total RNA prepared from *C. japonica* cells using RNeasy Plant Mini Kit (Qiagen) was reverse-transcribed with M-MLV reverse transcriptase (NEB). Nested PCR was carried out with Taq DNA polymerase (Takara), using the DNA-RNA hybrid as a template, and two sets of degenerate primers designed from the highly conserved

amino acid sequences in the nucleotide-binding fold (NBF) which is the domain responsible for the ATP-binding property of ABC proteins; Fwd-primer1, 5'-MARACIYTIGCHYTIGTIGG-3' (n- (Q/K)TTLALVG -c) and Rv-primer1, 5'-GCRCTXHTXGCYTCRTCXARXAG-3' (n- LLDEATSA -c): Fwd-primer2, 5'-GGITGYGGIAARWSIWSIGT-3' (n- GCGKS(T/S)V -c) and Rv-primer2, 5'-GCDATXCKYTGYTTYTGXCCXCC-3' (n- GGQKQRIA -c). As the PCR products, two 320 bp fragments for the NBF domain of MDR-like protein were isolated. Using two specific primers corresponding to the internal sequences of these DNA fragments, RT-PCR was performed again using a heat-stable DNA polymerase of high-fidelity KOD (TOYOBO), resulting in the amplification of two DNA fragments (2.2 kb) which was significantly similar to known MDR-like proteins. To obtain full-length cDNA clones, 5' and 3' RACE using a cDNA amplification kit (Clontech) were used. The nucleotide sequences of the full-length cDNAs, determined with a DSQ-2000 sequencer (Shimadzu), were designated *Cjmdr1* and *Cjmdr2*.

Organ-specific expression

Total RNAs were isolated from root, rhizome, petiole, leaf, peduncle, bud, flower, and seed of the intact plant of *C. japonica*. Aliquots of 10 μg of RNA were loaded on formamide-containing agarose gel, electrophoresised and blotted onto a nylon

membrane. As the hybridization probe, 3'-UTR of *Cjmdr1* (200 bp) and *Cjmdr2* (200 bp) were used. 18S rRNA was used as the load control.

Time-course of gene expression and berberine content.

Time-course of *Cjmdr1* expression in cultured cells of *Coptis japonica* was detected by Northern hybridization. The cultured cells were harvested 2, 5, 9, 14, 21 days after inoculation, from which poly $(A)^+$ RNA was isolated and loaded on formamide-containing agarose gel. As the hybridization probe, 3'-UTR of *Cjmdr1* (200 bp) and *Cjmdr2* (200 bp) were used. ATP synthase (β subunit) was used to analyze the loading amount. Cell growth and berberine content in the cells were also monitored.

For HPLC analysis, alkaloids were extracted from cells with HCl-acidified methanol until the cells lost their yellow color. The supernatant was subjected to HPLC analysis: mobile phase, 50 mM tartaric acid solution containing 10 mM SDS - acetonitrile - methanol (100 : 100 : 25); column, TSK-GEL ODS-80TM (TOSOH, 4.6 i.d. X 250 mm); temperature, 40°C; flow rate, 1.2 ml/min; detection, absorbance measured at 260 nm.

Chapter III

Functional analysis of CjMDR1 using *Xenopus* oocyte and yeast cells

Cultured *C. japonica* cells produce berberine and accumulate it exclusively in the vacuoles, and exogenously added berberine is also actively taken up by *C. japonica* cells to be accumulated in the vacuoles. These data suggested that two different transporters exist in *C. japonica* cells, one imports berberine to the cytosol at the plasma membrane, and the other exports berberine from the cytosol to the vacuole at the tonoplast membrane. In chapter II, I isolated *Cjmdr1* and *2* as potential berberine transporters from *C. japonica* cells using RT-PCR. Their organ specific expression suggested that the gene products of *Cjmdr1* and *2* are involved in the accumulation of berberine.

The best characterized plant ABC transporters to date are the AtMRP genes. These proteins are known to function as vacuolar sequesters of glutathionylated compounds, malonylated chlorophyll catabolites and glucuronides, which were demonstrated with yeast heterologous expression system (Martinoia *et al.*, 1993; Rea *et al.*, 1998; Theodoulou, 2000). Plant membrane transporters, however, also can be expressed in immature eggs (oocytes) from the South African clawed frog, *Xenopus*

laevis. The oocytes have fundamental sets of enzymes, proteins, and organelles, which all are required in the early developmental stages after fertilization has occurred.

In this chapter, I describe the functional analyses of CjMDR1 using heterologous expression systems; *Xenopus* oocyte and yeast cells. Characterization of CjMDR1 showed that this protein transported berberine as a substrate, and CjMDR1 did not show as broad a substrate specificity as other MDR-type ABC transporters, e. g., human MDR1.

Results

CjMDR1 functions as a berberine influx pump

I expressed CjMDR1 in *Xenopus* oocytes to characterize the function of this ABC protein. Oocytes injected with *Cjmdr1* mRNA or water as a negative control were placed in ND96 medium containing berberine, washed thoroughly, and then observed under fluorescent microscope. The *Cjmdr1*-injected oocytes showed higher fluorescence derived from berberine than the control oocytes, suggesting that CjMDR1 might function as a drug influx pump. This result was unexpected because MDR-type ABC proteins usually function as drug efflux pumps and no eukaryotic ABC transporter has yet been reported to be involved in drug influx.

Both microscopic observation (Fig. 3-1A) and OD_{420} measurement of berberine accumulation in oocytes (Fig. 3-1B) showed that the *Cjmdr1*-injected oocytes had a higher content of berberine than control oocytes. Confocal microscopic analysis indicated that berberine was mostly localized in the vegetative pole of the oocytes (Fig. 3-1A). Since CjMDR1 was suggested to have inward transport activity for berberine, the time-course of berberine uptake was monitored quantitatively by HPLC analysis after the lysis of oocytes (Fig. 3-2). *Cjmdr1*-injected oocytes showed a constant uptake of berberine from the medium in a time-dependent manner, whereas the berberine level in control oocytes remained nearly unchanged at a low level.

To examine whether this berberine uptake was dependent on CjMDR1, the effect of ATPase inhibitor and the intracellular ATP level were analyzed (Fig. 3-3). The uptake of berberine observed in *Cjmdr1*-injected oocytes without inhibitor was strongly inhibited to the control level by a membrane ATPase inhibitor, vanadate, and similar results were observed upon the addition of KCN, which causes ATP depletion. Effective ABC transporter-inhibitors, verapamil and nifedipine, which also function as Ca^{2+} channel blockers, inhibited berberine uptake by CjMDR1. Glibenclamide, another type of inhibitor for sulfonylurea receptor (SUR) and plant ABC transporters, also clearly inhibited berberine uptake (Fig. 3-3). The effects of bafilomycin A1 (0.1

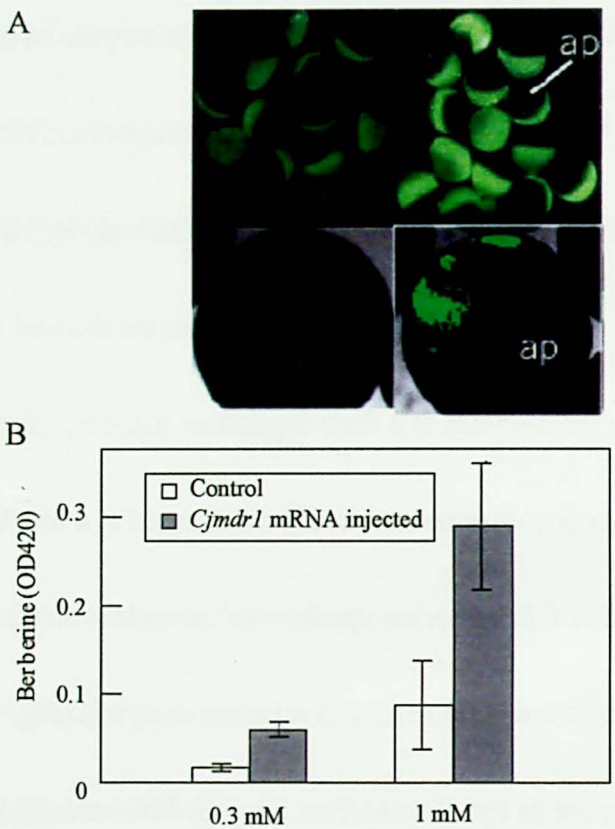


Fig. 3-1 Berberine accumulation in *Cjmdr1*-injected oocytes and a negative control.
(A) Fluorescent micrographs of *Cjmdr1*-injected oocytes (right) and water-injected oocytes (left). Both oocytes were incubated in the presence of 1 mM berberine in ND96 medium for 1 h. Bottom photographs were taken using a confocal microscope. Ap, animal pole.
(B) Amounts of berberine accumulated in *Cjmdr1*-injected or water-injected oocytes, which were incubated in 0.3 or 1.0 mM berberine medium. Amounts of berberine were estimated based on absorption at 420 nm.

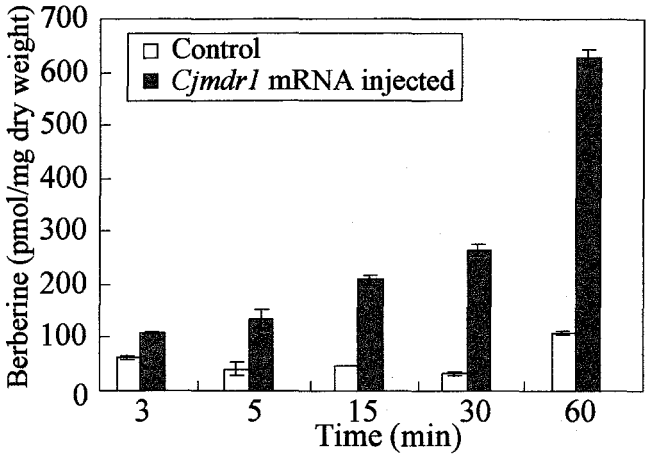


Fig. 3-2. Time-course of berberine influx into oocytes injected with *Cjmdr1* mRNA. *Cjmdr1*-injected and water-injected oocytes were incubated in berberine medium (1mM), and sampled at the times indicated for transfer into lysis buffer. The lysate was lyophilized and dissolved in 50% methanol to be analyzed by HPLC equipped with a photodiode array detector. The quantity of berberine was calculated from the peak area.

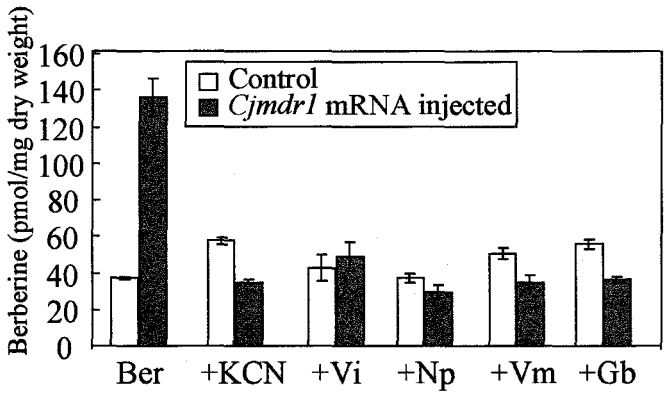


Fig. 3-3. Effects of inhibitors commonly used for ABC transporters on berberine uptake in *Cjmdr1*-injected oocytes. Amounts of berberine taken up in *Cjmdr1*-injected and water-injected oocytes after incubation in medium containing 1 mM berberine in the presence of inhibitor. Ber, no addition of inhibitor; KCN (1 mM); Vi, vanadate (1 mM); Np, nifedipine (100 μM); Vm, verapamil (100 μM); Gb, glibenclamide (100 μM).

μM), a specific V-type ATPase inhibitor, and NH_4^+ (5 mM), which destroy ΔpH across the membrane, were also tested, but they did not significantly inhibit berberine uptake, i.e. less than 12% inhibition for both.

To characterize CjMDR1 activity, the berberine content in oocytes which were loaded with berberine and then incubated in fresh medium without berberine was quantitatively analyzed (Fig. 3-4). *Cjmdr1*-injected oocytes retained a clearly higher berberine level than the negative control, and this berberine-retaining activity dramatically decreased when cells were treated with the ABC transporter-inhibitors, verapamil and glibenclamide, indicating that berberine was actively retained by CjMDR1.

Kinetic analysis of berberine uptake by *Cjmdr1*-injected oocytes revealed that K_m value and V_{max} were 54.6 ± 5.5 μM and 0.75 ± 0.02 nmol/mg protein/10 min, respectively (Fig. 3-5). The K_m value of CjMDR1 for berberine was similar to, whereas its V_{max} was *ca.* 10-fold lower than, that of AtMRP1 for glutathione conjugate of dinitrophenol (Lu *et al.*, 1998).

Other possible substrates of CjMDR1

I examined whether or not CjMDR1 was exclusively specific to berberine, by incubating oocytes with other natural compounds, e. g. reticuline, sanguinarine, quinine,

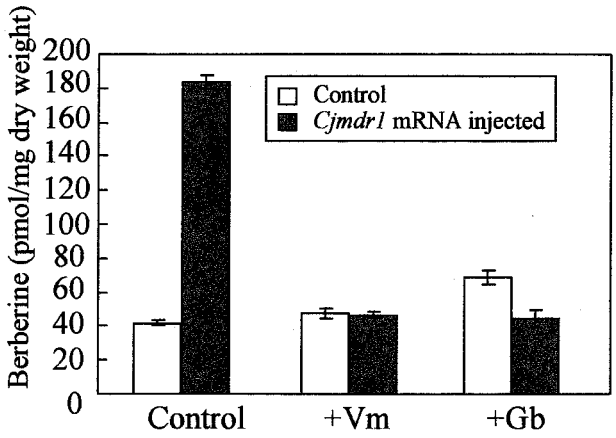


Fig. 3-4. Effects of inhibitors commonly used for ABC transporters on berberine uptake in *Cjmdr1*-injected oocytes. Berberine retained inside *Cjmdr1*-injected oocytes incubated in fresh medium after loading with berberine. Inhibitors were added 15 min before the oocytes were transferred from berberine-loading medium to fresh medium without berberine. Vm, verapamil (100 μM); Gb, glibenclamide, (100 μM).

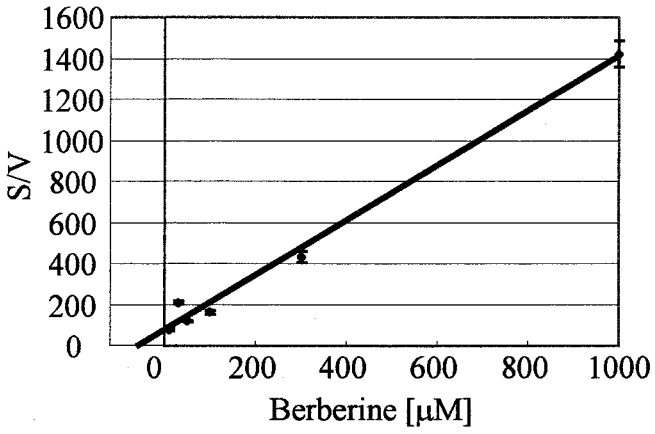


Fig. 3-5 Uptake of berberine into *Cjmdr1*-injected oocytes displays Michaelis-Menten-type saturation kinetics. Representative saturation experiments are illustrated as a Hanes-Woolf plot. *Km* value measured in three independent experiments ranged between 10 and 1000 μM.

and quercetin (Fig. 3-6). However, I didn't observe any difference in the uptake of these compounds between *Cjmdr1*-injected oocytes and the control except reticuline, a precursor of berberine, whose uptake was slightly higher in *Cjmdr1*-oocyte only at high concentration as 1 mM.

I also investigated the substrate specificity by estimating drug sensitivity in yeast transformant (Fig. 3-7). CjMDR1 was expressed with a shuttle vector, pDR196, with which a foreign gene is constitutively expressed by PMA1 promotor (Rentsch *et al.*, 1995), in yeast *S. cerevisiae*, strain AD12345678, which lacks major yeast ABC transporter-encoding genes that confer multidrug resistance (Decottignies *et al.*, 1998). The same yeast strain transformed with the empty vector was used as a negative control. As shown in Fig. 3-7, the CjMDR1 transformant appeared to be more sensitive to berberine, although the difference was rather small. A larger difference in drug sensitivity between CjMDR1 transformant and the control was found with 4-nitroquinoline *N*-oxide (4-NQO), a substrate of yeast PDR-type ABC transporter, SNQ2 (Servos *et al.*, 1993). This data suggests that 4-NQO was recognized as a substrate of CjMDR1 and taken up into cells, and the sensitivity of yeast was increased. However, cycloheximide, a substrate of the yeast PDR-type ABC transporter, PDR5 (Hirata *et al.*, 1994), showed no clear difference between yeast cells, indicating that

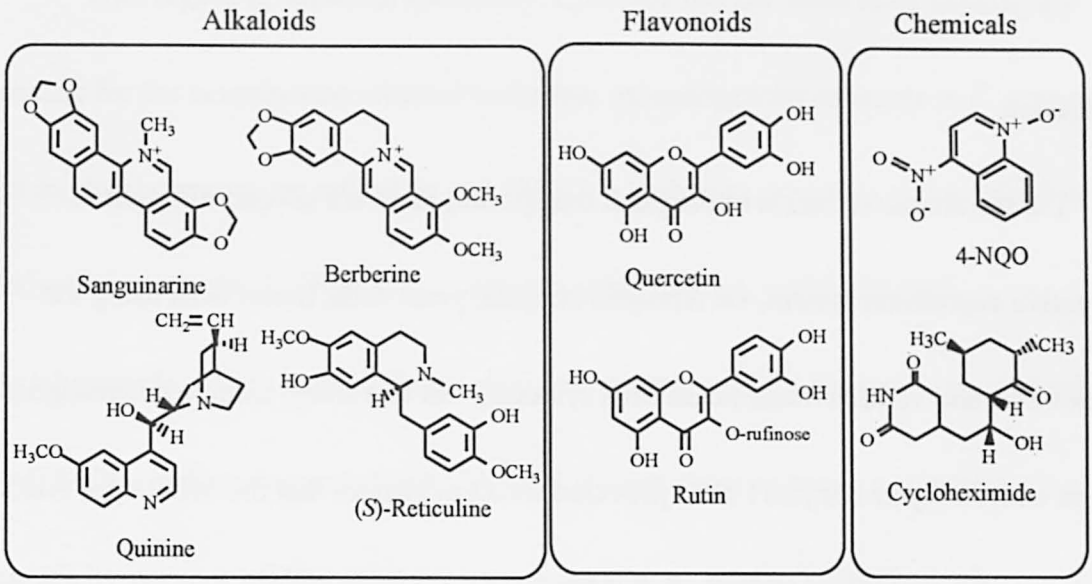


Fig. 3-6 Structures of alkaloids, flavonoids, and chemicals tested as CjMDR1 substrates. Berberine, (*S*)-reticuline, and 4-NQO were recognized as substrate by CjMDR1.

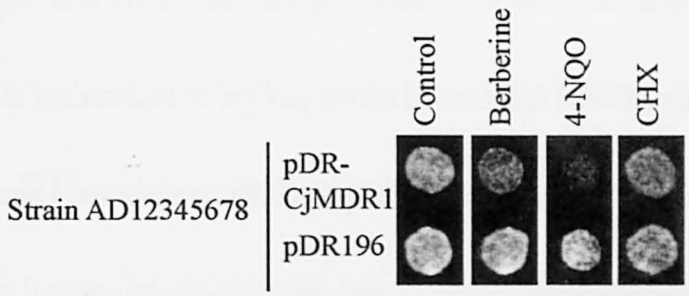


Fig. 3-7. Drug sensitivity of yeast CjMDR1 transformant.
Yeast strain AD12345678 was transformed with pDR196 (empty vector) or pDR-CjMDR1. Each transformant was pre-cultured overnight in SD medium (-uracil). The cultures were diluted to A600=0.5. Five μl was spotted onto an SD medium (-uracil) plate containing berberine (3.5 μM), 4-NQO (4-nitroquinoline N-oxide) (0.11 μg/ml) or CHX (cycloheximide) (0.0175 μg/ml) and incubated for 44 h at 25°C.

CjMDR1 had relatively strong substrate specificity.

Discussion

For the characterization of the plant transporter, there are several different alternative expression system, i.e. *Xenopus* oocytes, yeast cells, insect cells using the baculovirus, and various mammalian cells systems. In this study, using a heterologous system with *Xenopus* oocytes I have provided direct evidences that the MDR-type ABC transporter, CjMDR1, uptook berberine as the native substrate (Fig. 3-1~3-4). Thus far, all characterized mammalian ABC transporters which are localized in the plasma membrane show excretion activity for substrates, e. g. human MDR1 and members of the MRP subfamily in animal cells function as efflux pumps of anti-cancer drugs to exclude them from cytosol. Tonoplast-localized plant ABC transporters such as plant MRP proteins export glutathione conjugates of xenobiotics and chlorophyll catabolite from cytosol into the vacuolar matrix. While such inward transport of substrates from outside to inside of the cell by ABC transporters has often been reported in prokaryotic organisms, this is the first example of eukaryotic ABC protein functioning as an influx pumps. Since this transport activity was clearly observed in both *Xenopus* oocyte and yeast, I supposed that CjMDR1 was sorted to plasma membrane and integrated correctly

facing two NBF domains to cytosol space.

With regard to substrate specificity, CjMDR1 did not seem to be exclusively specific for the isoquinoline alkaloid berberine, an endogenous substrate in *C. japonica*, but might also recognize reticuline and 4NQO, a berberine precursor and a quinoline derivative, respectively (Fig. 3-7). Sanguinarine, quinine, quercetin, and cycloheximide, which have different chemical structures, did not seem to be recognized by CjMDR1, which suggested that CjMDR1 did not show as broad a substrate specificity as other MDR-type ABC transporters, e. g., human MDR1.

Plant ABC transporters have become one of the most active research fields in recent years. There have been many excellent studies to elucidate the function of MRP subfamily members (Lu *et al.*, 1998; Liu *et al.*, 2001; Tommasini *et al.*, 1998; Gaedeke *et al.*, 2001), PDR subfamily protein (Jasinski *et al.*, 2001; van den Brule *et al.*, 2002), and the unique peroxisome-localized ABC protein (Hayashi et al., 2002; Zolman *et al.*, 2001). However, little is known about the endogenous substrates of these ABC proteins, and the MDR subfamily in particular, such as PMDR1 of potato (Wang *et al.*, 1997), HvMDR2 of barley (Davies *et al.*, 1997), and TaMDR1 of wheat (Sasaki *et al.*, 2002), is still open for investigation, except for AtPGP1 (Sidler *et al.*, 1998) that is one of the 22 members in *Arabidopsis* MDR-type proteins. The *Arabidopsis* ABC protein

that is most similar to CjMDR1 is AtPGP4, whose function is still unknown. Present findings may provide some new clues to clarify their functions and physiological roles in plant.

Materials and Methods

Chemicals.

Unless otherwise stated, the purest chemicals were obtained and used as described in Chapter I.

Oocyte Isolation and Injection.

An African clawed frog, *Xenopus laevis,* was anesthetized by hypothermia (20 min in ice-cold water). Part of one ovary was removed, cut into small portions and placed in OR2 medium (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES-NaOH, pH 7.4). Oocytes were prepared as described previously (Dascal, 1987), and mature stage VI oocytes were injected with *Cjmdr1* mRNA (50 nl, 1 ng/nl), or sterile water for control oocytes, using a micropipette attached to a hydraulic microinjector (Nanoject, Drummond, Broomall, USA). Glass micropipettes with a tip diameter of 10-30 μm were pulled on a Narishige PE6 pipette puller (Tokyo, Japan). Injected oocytes were kept at 18°C for 3 days in ND96 medium (96 mM NaCl, 2 mM

KCl, 1.8 mM $MgCl_2$, 1 mM $CaCl_2$, 2.5 mM Na-pyruvate, 5 mM HEPES-NaOH, pH 7.4), which ensured 90% survival. Damaged oocytes were separated from intact oocytes bearing a clear brown pole animal hemisphere and a distinct equator line.

Expression of CjMDR1 in *Xenopus* Oocytes.

Cjmdr1 cDNA (4.2 kb) was subcloned in a pCF3 vector derived from the *Xenopus* oocyte expression vector pBSTA, kindly provided by Dr. Goldin (University of California at Irvine), after slight modifications. The pBSTA vector contains a T7 promoter followed by 5' non-coding β-globin sequences, a single *Bgl* II site for insertion of exogenous DNA, 3' non-coding β-globin sequences, a poly(A) tail and a polylinker so that the plasmid would be linearized before *in vitro* transcription (Shih *et al.*, 1998). The addition of 5' and 3' non-coding sequences from the β-globin gene greatly increases the expression of exogenous proteins in oocytes (Krieg and Melton, 1984). *In vitro* transcription followed by capping of the RNA was performed with the plasmid (1 μg) linearized at the unique site *Asc* I using the T7 mMESSAGE mMACHINE™ kit (Ambion, USA). The template DNA was degraded by DNase I, and *Cjmdr1* mRNA was purified on formaldehyde gel and quantified.

Measurement of Berberine Transport in Oocytes.

All transport experiments were conducted 2-3 days after the injection of oocytes at 18°C. Fluorescence in the oocytes derived from berberine was monitored using a fluorescence microscope (excitation 420 nm / emission 517 nm). To quantitatively determine the influx of berberine into oocytes, water- and *Cjmdr1*-injected oocytes (15-20 oocytes per batch) were incubated in the presence of berberine (0.3 mM or 1 mM) for 1 h, unless otherwise specified. In time-course experiments, oocytes exposed to 1 mM berberine were sampled periodically (3, 5, 15, 30, 60 min), lysed in ND96 (pH 5), and lyophilized, and 2 mg of dried material was used for HPLC analysis. Efflux experiments were performed by transferring oocytes loaded with berberine into fresh ND96 solution after careful washing to remove extracellular berberine. Inhibitors were added to the oocyte suspension and then incubated for 15 min at 4°C. After 60 min of efflux, the remaining berberine in the oocytes was quantitatively analyzed by HPLC.

The ATP-dependent uptake of berberine in *Cjmdr1*-injected oocytes was also investigated by depleting ATP from oocytes with a metabolic inhibitor (1 mM KCN), by suppressing the ATPase activity of the transporter (1 mM vanadate) or by applying classical blockers of ABC transporters (100 μM nifedipine, verapamil, and

glibenclamide).

HPLC Conditions.

For HPLC analysis, dried oocytes were suspended in 50% methanol (0.01 N HCl) by vigorous pipetting, and centrifuged at 14,000 rpm for 10 min. The supernatant was subjected to HPLC analysis: mobile phase, 50 mM tartaric acid solution containing 10 mM SDS - acetonitrile - methanol (100 : 130 : 33); column, TSK-GEL ODS-80TM (TOSOH, 4.6 i.d. X 250 mm); temperature, 40°C; flow rate, 1.2 ml/min; detection, absorbance measured at 260 nm using a photodiode array detector.

Expression of CjMDR1 in Yeast.

Cjmdr1 cDNA (4.2 kb) was subcloned into yeast expression vector pDR196 (Rentsch *et al.*, 1995), kindly provided by Dr. W. Frommer (University of the Tübingen), at the *Not* I site. The resulting plasmid, pDR-CjMDR1, was used to transform strain AD12345678 (*yor1*Δ, *snq2*Δ, *pdr5*Δ, *pdr10*Δ, *pdr11*Δ, *ycf1*Δ, *pdr3*Δ, *pdr15*Δ) (Decottignies *et al.*, 1998) by the lithium acetate method (Ito *et al.*, 1983), and then selected by SD medium (-uracil). The yeast transformant was cultured in 100 ml of SD medium (-uracil), and membrane proteins were extracted for the detection of recombinant CjMDR1 as described previously (Yazaki *et al.*, 2002).

Drug Sensitivity Assay.

The drug sensitivity of yeast transformants was tested by spotting SD- cultures (-uracil) onto agar plates containing various compounds. Five μl of the transformant diluted to same density A_{600}=0.5 was spotted onto each plate and growth was monitored after incubation of the cells for 44 h at 25°C. The drugs, berberine and cycloheximide, used in this study were dissolved in water, while 4-nitroquinoline *N*-oxide was dissolved in acetone.

Chapter IV

Subcellular and cell-specific localization of CjMDR1

In chapter III, I analyzed the function of CjMDR1 using *Xenopus* oocyte and yeast cells. CjMDR1 transported berberine as a substrate in inward direction. These results suggested that the localization of this protein should be plasma membrane, and then that this protein was involved in the uptake of exogenously added berberine to the culture medium into *C. japonica* cells. To confirm this hypothesis, I analyzed the localization of CjMDR1 by conventional membrane separation methods. In this chapter, I describe the subcellular and cell-specific localization of CjMDR1 and the model of the physiological function of CjMDR1 *in planta*.

Results

CjMDR1 was localized at the plasma membrane

To understand the physiological function of CjMDR1 *in planta*, its subcellular localization was determined. For this purpose, I prepared polyclonal antibodies against a specific oligopeptide of CjMDR1 as described in the Materials and Methods, and the reactivity of these antibodies to CjMDR1 was examined both in a heterologous

expression system with yeast and in intact *C. japonica* membranes (Fig. 4-1A). In plant membranes, anti-CjMDR1 antibodies detected a band at 140 kDa, whereas no detectable protein was observed in the soluble fraction. A band at 140 kDa was also detected in the membranes of yeast cells transformed with *Cjmdr1* cDNA, but was absent in the negative control using the empty vector.

Using these antibodies, I determined the subcellular localization of CjMDR1 by two different methods. First, microsomal membranes were fractionated on sucrose density gradients and then analyzed by immunoblotting (Fig. 4-1B). CjMDR1 and the plasma membrane H^+-ATPase were co-fractionated to give a peak at the interface between 30 and 40% sucrose, whereas membranes containing luminal binding protein (BiP), a marker of endoplasmic reticulum (Haas *et al.*, 1994), and vacuolar H^+-pyrophosphatase (V-PPase), a marker of tonoplast membrane (Uemura *et al.*, 2002), sedimented at positions different from CjMDR1 (Fig. 4-1B). These results suggested that CjMDR1 was associated with the plasma membrane and not with the tonoplast membrane or endoplasmic reticulum.

The localization of CjMDR1 in the plasma membrane was also supported by an analysis using the aqueous two-phase partitioning method (Larsson et al., 1987), by which the plasma membrane was separated from other intracellular membranes of *C.*

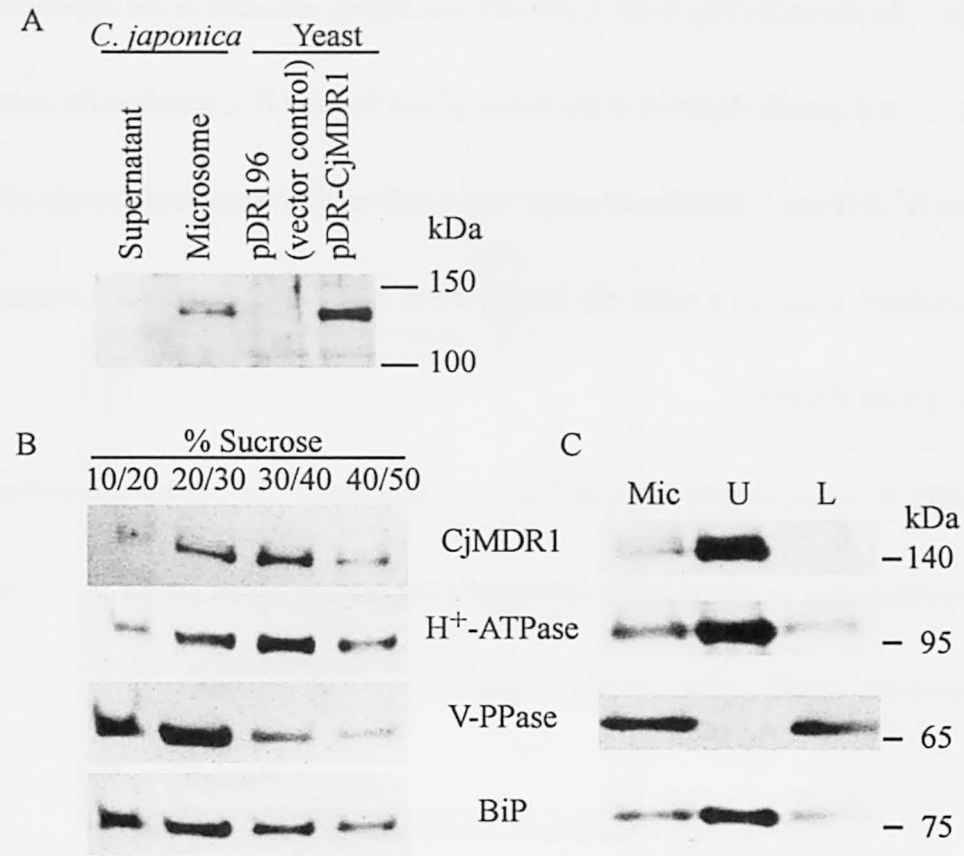


Fig. 4-1 Immunodetection of CjMDR1 in *C. japonica* cells and yeast cells.

(A) Soluble and membrane (microsomal fraction) proteins (10 μg per lane) of *C. japonica* cells and membrane proteins (10 μg per lane) of yeast expressing CjMDR1 or empty vector were separated by SDS-PAGE and blotted onto a PVDF membrane. The membrane was probed with anti-CjMDR1 polyclonal antibodies.

(B) Immunodetection of CjMDR1 in *C. japonica* plasma membranes. Fractionation of total microsomes from *C. japonica* cells on a non-continuous sucrose gradient consisting of 10, 20, 30, 40, and 50% (w/v) sucrose. Membrane fractions were collected from the interfaces between different sucrose concentrations. Proteins from each interface were immunodetected with anti-CjMDR1 antibodies. Blots were probed with antibodies raised against CjMDR1, plasma membrane H^+-ATPase (H^+-ATPase), vacuolar H^+-pyrophosphatase (V-PPase), and endoplasmic reticulum BiP (BiP).

(C) Microsomal membranes (M) from *C. japonica* cells were fractionated by the aqueous two-phase partitioning method into an upper phase (U) enriched with plasma membranes and a lower phase (L) containing intracellular membranes. Proteins from each fraction (10 μg per lane) were immunoblotted. The same membrane was reprobed with antibodies against the indicated marker proteins.

japonica. As shown in Fig. 4-1C, CjMDR1 was highly enriched in the upper-phase fraction (U) and greatly depleted in the lower-phase fraction (L), which is the same pattern as H^+-ATPase. Membrane purity was confirmed by immunodetection of the marker proteins, although a relatively large portion of ER marker was also detected in the upper-phase fraction.

These results indicated that CjMDR1 was localized at the plasma membrane, and that this protein was involved in the uptake of exogenously added berberine to the culture medium into *C. japonica* cells (Fig. 4-2).

Cell-specific accumulation of berberine in the Coptis japonica *tissue*

To identify the cell-specific accumulation of alkaloid in the rhizome, several observations were carried out on cross-sections of *C. japonica* tissues (Fig. 4-3). In the bright field, vascular bundle of leaf stalk showed pale yellow, whereas outer cortex and bast fiber of rhizome and epidermis of root showed strong yellow pigmentation. On the other hand, when samples were observed under UV irradiation to emit yellow fluorescence of berberine, strong yellow-green fluorescence was observed for all vascular bundle and bast fiber of all tissues. Since the yellow fluorescence of pure berberine differs from that of the putative auto fluorescence in the cell wall of vascular

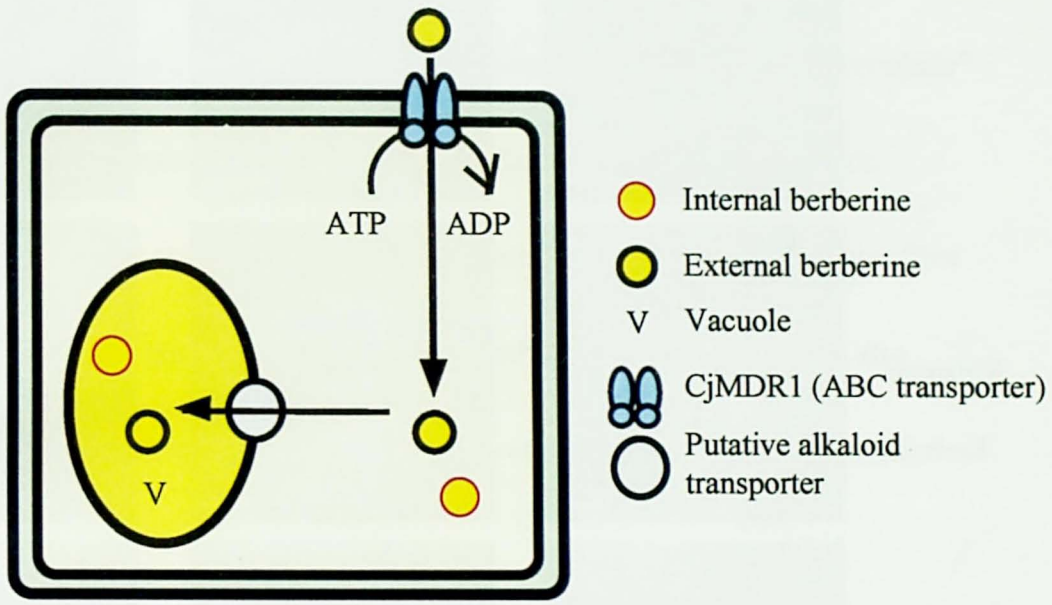


Fig. 4-2 A model for active transport of berberine in *Coptis japonica* cell
Berberine, that is exogenously added to the medium, is actively taken up by CjMDR1 which is localized at the plasma membrane of *C. japonica* cells. ATP hydrolysis is essential for berberine transport by CjMDR1.

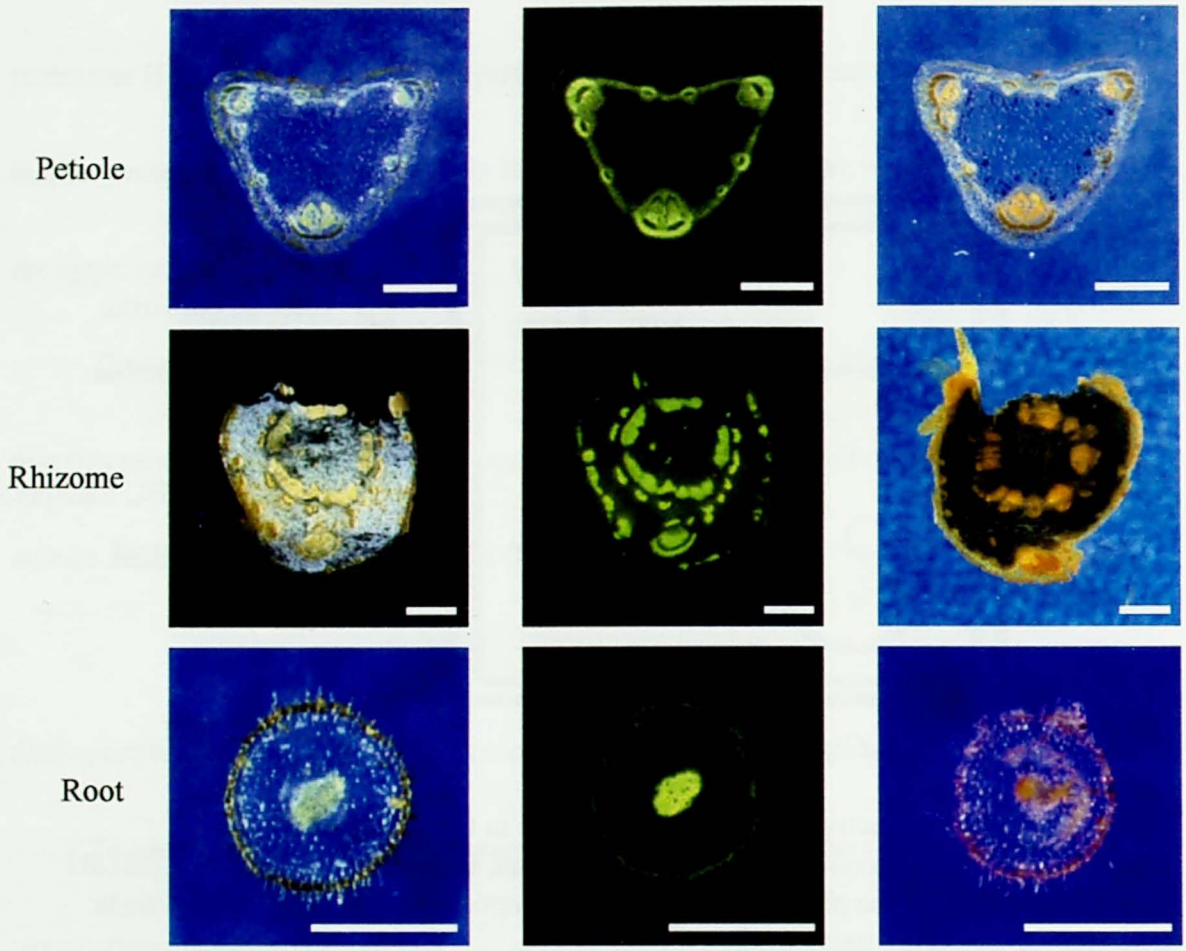


Fig. 4-3 Bright field and fluorescent micrographs of *Coptis japonica* tissues
Petiole, root, and rhizome sections were observed under bright (left) and fluorescent (center), and Dragendorff's reagent treated (right) conditions. Bars; 1mm.

tissues, and a blueish auto fluorescence is generally observed in such vascular tissues of plants, which accumulated phenolic compounds in the cell walls of these tissues, these characteristic yellow-green color might be the mixture of two fluorescent colors derived from phenolic compounds and berberine. The treatment with Dragendorff's reagent showed the low accumulation of alkaloid in vascular bundle of petiole and root. Whereas high alkaloid accumulation stained vascular bundle, epidermis and bast fiber in rhizome red, starch granules, highly accumulated in the cortex of rhizome, also turned purple-blue with iodine in Dragendorff's reagent, made identification of alkaloid difficult.

In situ localization of Cjmdr1 *and* Cjmdr2 *transcripts*

In Chapter II, Northern analysis showed that *Cjmdr1* and *2* mRNA were highly expressed in rhizome, where berberine is preferentially accumulated. To define the specific cell type in which these mRNA are accumulated, *in situ* hybridization experiments were performed using a digoxigenin-labeled antisense RNA probe prepared from the these cDNAs. The rhizome and the root, which correspond to the sink organ for berberine accumulation and the source organ for berberine biosynthesis, respectively, were sectioned and analyzed (Fig. 4-4~4-7). In the rhizome section,

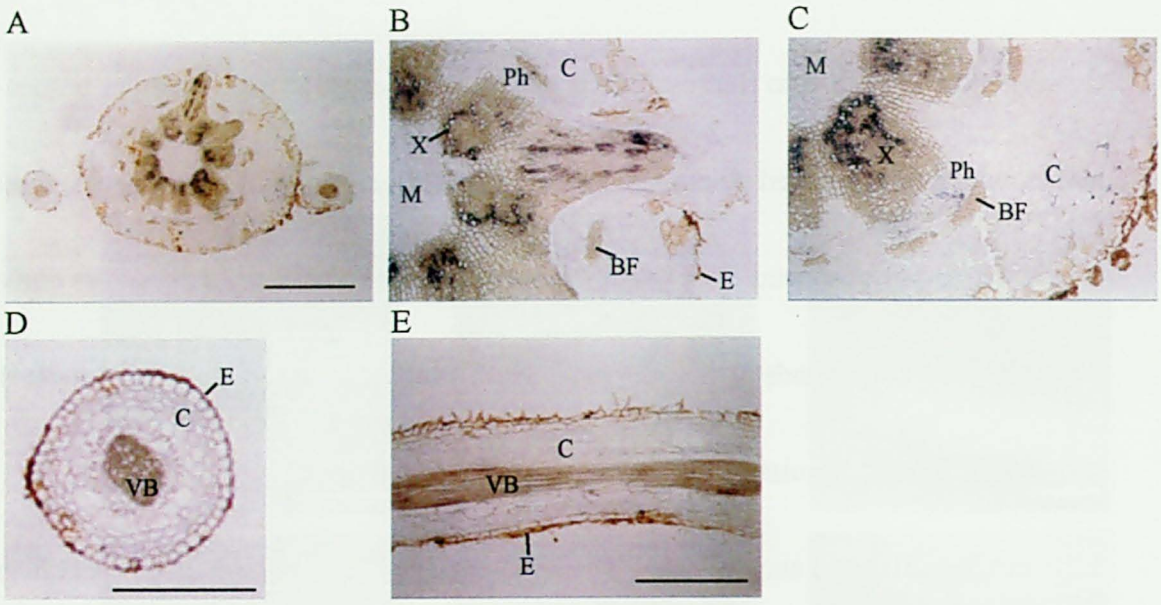


Fig. 4-4 *In situ* hybridization of *Cjmdr1* antisense in the rhizome and root of *C. japonica*.
(A, B, and C) *In situ* hybridization using a digoxigenin-labeled antisense probe in the rhizome.
(D, E) *In situ* hybridization using a digoxigenin-labeled antisense probe in the root.
BF, bast fiber; C, cortex; E, epidermis; M, medulla; Ph, phloem; VB, vascular bundle;
X, xylem. Bars, 500 μm.

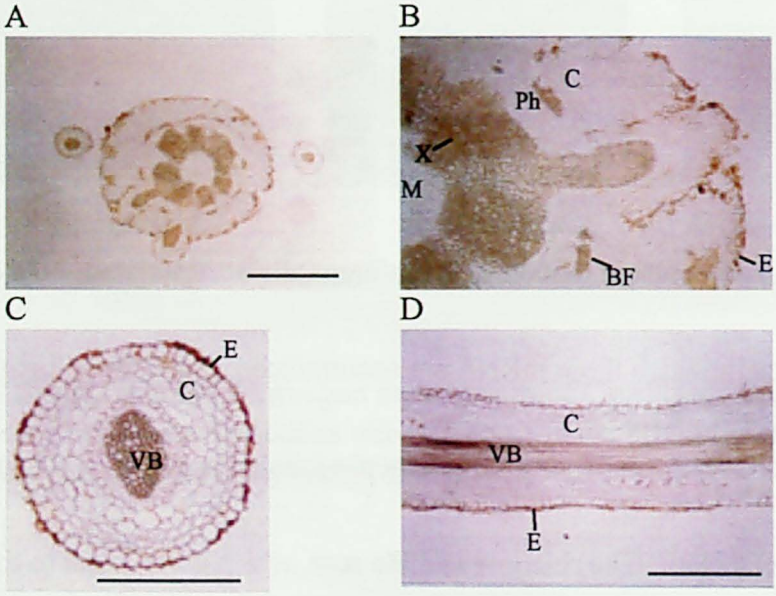


Fig. 4-5 *In situ* hybridization of *Cjmdr1* sense in the rhizome and root of *C. japonica*.
(A, B) *In situ* hybridization using a digoxigenin-labeled sense probe in the rhizome.
(C, D) *In situ* hybridization using a digoxigenin-labeled sense probe in the root.
BF, bast fiber; C, cortex; E, epidermis; M, medulla; Ph, phloem; VB, vascular bundle;
X, xylem. Bars, 500 μm.

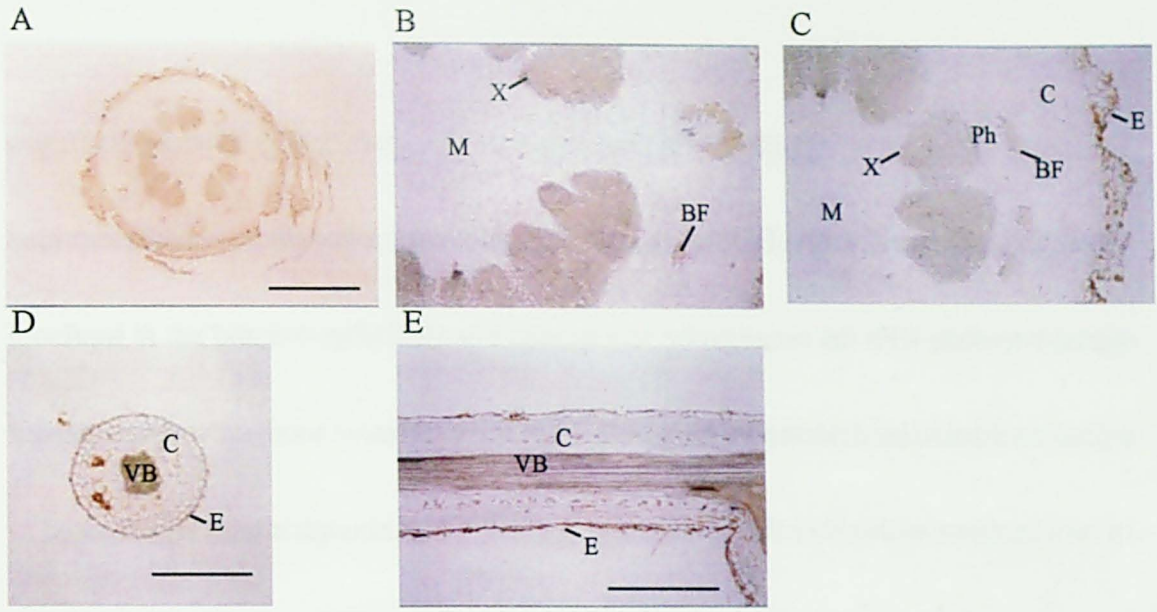


Fig. 4-6 *In situ* hybridization of *Cjmdr2* antisense in the rhizome and root of *C. japonica.*
(A, B, and C) *In situ* hybridization using a digoxigenin-labeled antisense probe in the rhizome.
(D, E) *In situ* hybridization using a digoxigenin-labeled antisense probe in the root.
BF, bast fiber; C, cortex; E, epidermis; M, medulla; Ph, phloem; VB, vascular bundle;
X, xylem. Bars, 500 μm.

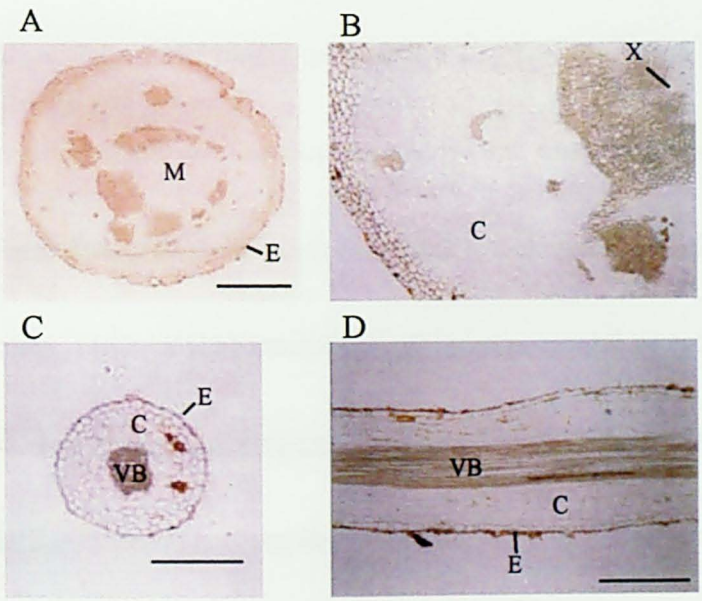


Fig. 4-7 *In situ* hybridization of *Cjmdr2* sense in the rhizome and root of *C. japonica.*
(A, B) *In situ* hybridization using a digoxigenin-labeled sense probe in the rhizome.
(C, D) *In situ* hybridization using a digoxigenin-labeled sense probe in the root.
BF, bast fiber; C, cortex; E, epidermis; M, medulla; Ph, phloem; VB, vascular bundle;
X, xylem. Bars, 500 μm.

strong signals were observed around the vascular tissue, particularly xylem cells (Fig. 4-4 A, B, and C, and 4-6A, B, and C), with the antisense probe, whereas no detectable signal was seen with the sense probe as a negative control (Fig. 4-5, 4-7). A weak signal could also be detected in the cortex. However, almost no signals were detected in root sections with either the antisense or sense probe, although a faint signal was sometimes observed in the vascular tissue (Fig. 4-4~4-7). These results suggest that an ABC transporter, CjMDR1, might participate in the unloading of berberine, which is transported from root tissue, in xylem tissues of the rhizome.

Discussion

Using sucrose density gradients and aqueous two-phase partitioning method, I showed that CjMDR1 was clearly localized in the plasma membrane of *C. japonica* cells. In chapter II, I demonstrated the isolation of cDNAs of *Cjmdr1* and *2* as potential berberine transporters from berberine-producing cultured cells of *C. japonica*, and found that these molecular species were expressed preferentially in the rhizome of the intact plant, where the main alkaloid berberine is specifically accumulated (Fig. 2-5). In Chapter III, I have provided direct evidence that one of these MDR-type ABC transporters, CjMDR1, uptook berberine using a heterologous system with *Xenopus*

oocytes (Fig. 3-2). In the present chapter, I showed that CjMDR1 as well as CjMDR2 was specifically expressed in xylem tissue in rhizome (Fig. 4-4, 4-6) and CjMDR1 localized in the plasma membrane of *C. japonica* cells (Fig. 4-1). All of these findings were consistent with the hypothesis that this ABC protein was involved in the unloading of berberine in the rhizome, i.e. berberine was biosynthesized in root tissues and translocated upward probably via xylem transport, while in the rhizome the berberine molecule was trapped by plasma membrane-localized CjMDR1 and accumulated in the rhizome tissue (Fig. 4-8). The function of CjMDR2 is yet unclear, however, CjMDR2 had high similarity to CjMDR1 on the amino acid level and *Cjmdr2* expression pattern was nearly identical with that of *Cjmdr1*, suggesting that CjMDR2 also functions as berberine transporter.

It is not yet clear why *C. japonica* had specific tissues for berberine biosynthesis and accumulation, but feedback inhibition of biosynthetic enzyme by berberine, the end product, might be one possible explanation (Sato F. *et al.*, 1993). The accumulation of berberine in rhizome would also be beneficial for the plant, because the rhizome is the sink organ for starch (Fig. 4-3), which is an attractive carbon source for soil bacteria and fungi, but berberine may protect this tissue from the attack by these pathogens. Elicitor-induced induction of berberine biosynthesis also supports the physiological

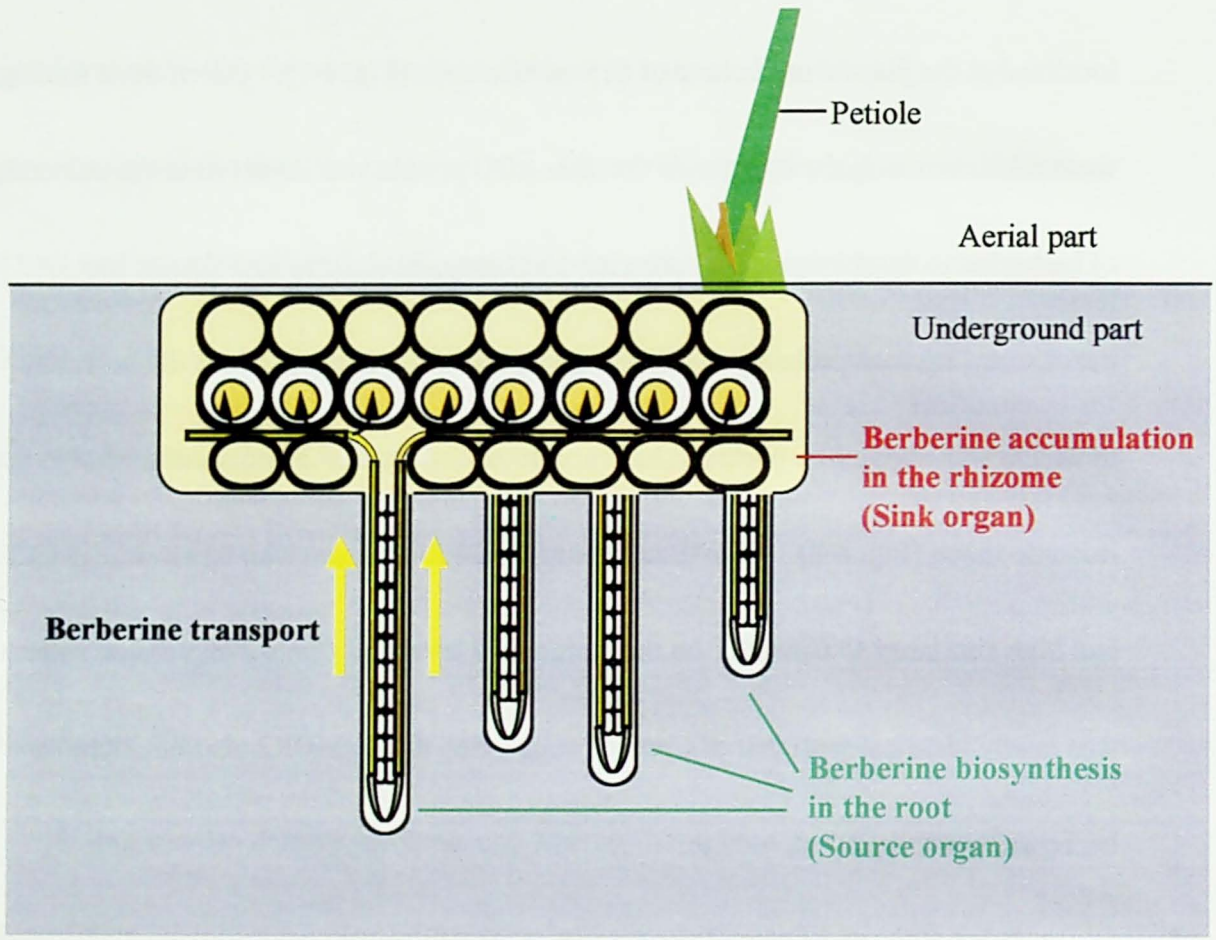


Fig. 4-8 A model of berberine accumulation in intact *Coptis japonica*.
Berberine, synthesized in the root, is translocated to the rhizome via xylem.
CjMDR1, localized in the plasma membrane, is involved in the berberine uptake and accumulation in the rhizome.

function of berberine and related alkaloids as phytoalexins (Funk *et al.*, 1987). The compartmentation of biosynthetic cells/tissues and sink cells/tissues is generally observed for plant secondary metabolites, such as tropane alkaloids, nicotine alkaloids and so on (Hashimoto and Yamada, 1994; Hartmann, 1999). The transport system demonstrated in *C. japonica* plant by this study may be a model for understanding such a transport mechanism in plant body.

Materials and Methods

Chemicals.

Unless otherwise stated, the purest chemicals were obtained and used as described in Chapter I.

Peptide Antibody Against CjMDR1.

A KLH (keyhole limpet hemocyanin) conjugate of an oligopeptide of CjMDR1, at position 378 (n- CSY DTS GHK SDD IRG D –c), was injected into rabbit according to the standard protocol (Sawady Technology, Tokyo, Japan). After the sixth boost, the antiserum was recovered and used for immunoblot analysis without further purification.

Isolation of Plasma Membranes.

Plasma membranes were purified from a microsomal fraction of *C. japonica* cells

by partitioning in an aqueous polymer two-phase system as described previously (Larsson et al., 1987). The upper phase, in which the plasma membrane was enriched, and lower phase were used for immunoblot analyses.

For sucrose gradient fractionation, a microsomal pellet was resuspended in a small volume of the resuspension buffer (10 mM Tris-HCl, pH 7.6, 1 mM DTT, 10% (v/v) glycerol, and 1 mM EDTA) and layered onto a non-continuous sucrose gradient from 10 to 50% (w/v) in the centrifugation buffer (10 mM Tris-HCl, pH 7.6, 1 mM DTT, and 1 mM EDTA). The gradient was then centrifuged at 100,000 g for 2 h and the membranes were collected from the interface between different sucrose concentrations.

Protein Gel Blotting.

For immunoblotting, proteins were denatured in the denaturation buffer (10 mM Tris-HCl, pH 8.0, 40 mM DTT, 1 mM EDTA, 10% (w/v) sucrose, 10 μg/ml Pyronin Y, and 2% (w/v) SDS) for 10 min at 50°C, subjected to SDS-PAGE (7% gel) and then transferred to an Immobilon™ polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was treated with BlockAce (Dainippon-seiyaku, Osaka, Japan) for blocking, incubated with primary antibodies, and subsequently with the secondary horseradish peroxidase-conjugated anti-rabbit antibodies by standard procedures. The band was visualized by chemiluminescence (PerkinElmer, USA). Antibodies used for

immunodetection were against CjMDR1, vacuolar H^+-pyrophosphatase from *Arabidopsis* (V-PPase), endoplasmic reticulum BiP (BiP), and plasma membrane H^+-ATPase (H^+-ATPase).

***In Situ* Hybridization.**

Root and rhizome were fixed on ice in freshly prepared solutions of 2 and 4% formaldehyde in 50% ethanol and 10% acetic acid, respectively, for 3 h. The samples were dehydrated through an ethanol series, and embedded with Paraplast plus (OXFORD). Sections 10- to 20-μm thick prepared with a microtome (RM 2155, Leica) were hybridized with the digoxigenin-labeled antisense or sense RNA probe of *Cjmdr1* or *2*, which is a 200 bp fragment containing the 3'-untranslated region. *In situ* hybridization and the detection procedures were performed as described previously (Schmelzer *et al.*, 1989, Yamada *et al.*, 1995).

Summary

Chapter I

Treatment with several inhibitors suggested that berberine uptake depended on the ATP level. Some inhibitors of human MDR1 showed strong inhibitory effect on the berberine uptake, whereas buthionine sulfoximine, a specific inhibitor for glutathione biosynthesis, and vacuolar ATPase inhibitor, bafilomycin A1, had only little effect. These results suggested that an MDR-like protein was involved in the uptake and accumulation of berberine by the cells.

Vanadate-induced nucleotide trapping experiments suggested the existence of ABC transporters in *C. japonica* cell. Two of the three bands showed the interaction with berberine, which suggested that they were involved in the transport of berberine in this plant cell.

Chapter II

Two full-length cDNAs, *Cjmdr1* and *Cjmdr2*, which belonged to the *mdr* gene family, were isolated by nested RT-PCR and RACE from *C. japonica* cells. *Cjmdr1* and *Cjmdr2* were ca. 4.2 kb and had ORFs of 1289 a.a. and 1292 a.a., respectively. Northern analysis in the intact plant showed a clear preference in their expressions to

the rhizome, where alkaloids are highly accumulated compared to other organs.

Chapter III

Functional analysis of CjMDR1 using a *Xenopus* oocyte expression system showed that CjMDR1 transported berberine in inward direction, resulting in a higher accumulation of berberine in *Cjmdr1*-injected oocytes than in the control. Typical inhibitors of ABC proteins, such as vanadate, nifedipine, and glibenclamide, as well as ATP depletion by KCN clearly inhibited the CjMDR1-dependent berberine uptake, suggesting that the transport property of CjMDR1 was that of an ABC transporter. The kinetic analysis of berberine uptake by *Cjmdr1*-injected oocytes revealed that K_m value and V_{max} were 54.6 ± 5.5 μM and 0.75 ± 0.02 nmol/mg protein/10 min, respectively. With regard to substrate specificity, CjMDR1 was highly specific to berberine, an endogenous substrate in *C. japonica*, but did not seem to be exclusively specific for this alkaloid and might also recognize reticuline and 4NQO, a berberine precursor and a quinoline derivative, respectively.

Chapter IV

Peptide antibodies against CjMDR1 specifically recognized this polypeptide, and

detected a band at 140 kDa in intact *C. japonica* membranes. Using sucrose density gradients and the aqueous two-phase partitioning method, I showed that CjMDR1 was localized in the plasma membrane of *C. japonica* cells. *In situ* hybridization indicated that *Cjmdr1* as well as *Cjmdr2* mRNAs was expressed preferentially in xylem tissues of the rhizome, where berberine is highly accumulated. Whereas the biosynthetic genes of berberine are specifically expressed in root tissue, in which only low level of alkaloid is observed.

These findings strongly suggested that CjMDR1 was involved in the translocation of berberine from the root to the rhizome. In the rhizome, the berberine molecule, which is translocated upward probably via xylem transport, is trapped by plasma membrane-localized CjMDR1 and accumulated in the rhizome tissue. This is the first example of an eukaryotic ABC transporter functioning as influx pump and also the first demonstration that an ABC transporter plays a role in unloading process of an endogenous metabolite.

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